Regulation of T cell activity in virus infections

by

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Statement

The FMF sorting experiments in Chapter 5 were performed in collaboration with Felicity Lynch and the in vivo adoptive cell transfer experiments in Chapter 4 with Prof. P.C. Doherty and Dr. Jane Allan. Otherwise, the work described in this thesis was carried out by the candidate.

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Abstract

The studies presented in this thesis have examined the requirement for T cell activation in primary and secondary anti-viral T cell responses.

Subclinical infection of mice with vaccinia virus can be converted into lethal disease by prior treatment with the immunosuppressive drug, cyclophosphamide. The drug interferes with the development of the cytotoxic T cell reponse which is necessary for elimination of virus. Spleen cells from the immunosuppressed animals are characterized by a diminished capacity to proliferate and produce IL-2 in vitro upon mitogenic stimulation. CTL precursors from the treated mice can be stimulated by administering IL-2 in vivo or by culturing the cells in the presence of IL-2 in vitro. The results suggest that IL-2 produced by T helper cells is necessary for development of the primary anti-viral CTL response in vivo.

During the study of the requirements for activation of memory T cell, it was found that CTL primed in vivo with LCMV can be specifically reactivated with phorbol myristate acetate (PMA) and calcium ionophore (CaI) in the absence of added antigen. The optimal conditions of this stimulation were determined at bulk and clonal levels, and used in further experiments as a method for reactivation of memory CTL. The cytotoxic activity of primed CTLp stimulated with PMA and CaI was found to be IL-2 dependent although some proliferation occured in the absence of T helper cells or exogeneous IL-2. LCMV-specific memory cells are also characterized by the expression of the Ly 24 (Pgp 1) glycoprotein.

The in vitro restimulated secondary immune CTL response against a complex antigen consisting of mouse alloantigen ($H\text{-}2K^d$) and vaccinia virus has also been studied. It was found probable that distinct T cell clones at similar frequencies are responsible for the anti-allo and anti-viral immune responses.

The results discussed in this thesis support the theory of T helper - T cytotoxic cell interaction during the development of primary and secondary anti-viral immune responses and reveal that memory CTL differ from unprimed virus specific CTLp not only in frequency but also in

requirements for the stimulation of cytotoxic activity and by the appearance of a distinct surface marker, Ly 24.

Note

Much of the work described in this thesis has been published as listed below:

Doherty, P.C., Allan, J.E., Dixon, J.E., Tabi, Z. and Ceredig, R. **1986** Characteristics of the CSF inflammatory exudate in murine lymphocytic choriomeningitis. In: Proceeding of "Workshop on cellular and humoral components of CSF in Multiple Sclerosis" eds. Lowenthal, A. and Raus, J. Plenum Press. p 351.

Ceredig, R., Allan, J.E., Tabi, Z., Lynch, F. and Doherty, P.C. **1987** Phenotypic analysis of the inflammatory exudate in murine lymphocytic choriomeningitis. J. Exp. Med. 165:1539.

Tabi, Z., Allan, J.E., Ceredig, R. and Doherty, P.C. **1988** Lethal vaccinia infection in cyclophosphamide-suppressed mice is associated with decreased expression of Thy-1, Lyt-2 and L3T4 and diminished IL-2 production in surviving T cells. Immunol. 63:423.

Tabi, Z., Lynch, F., Ceredig, R., Allan, J.E. and Doherty, P.C. **1988** Virus-specific memory T cells are Pgp 1^{+} and can be selectively activated with phorbol ester and calcium ionophore. Cell. Immunol. 112: (in press)

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
Arm	Armstrong strain of LCMV
Au	arbitrary units
C	complement
Cal	Calcium ionophore
CMI	Cell mediated immunity
Con A	Concanavalin A
cpm	counts per minute
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
Cy	Cyclophosphamide
f	frequency
FACS	Fluorescence activated cell sorter
FCS	foetal calf serum
FIs	forward light scatter intensity
FMF	fluorescence microfluorometer
HBSS	Hank's buffered salt solution
i.c.	intracerebral
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
LAK	lymphokine activated killer cells
LCMV	lymphocytic choriomeningitis
LD_{50}	50% lethal dose
LU	lytic units
mAb	monoclonal antibody

MHC	Major histocompatibility complex
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
m.o.i.	multiplicity of infection
NK	natural killer cell
O.D.	optical density
PBS	phosphate buffered saline
PFU	plaque forming unit
PHA	phytohemagglutinin
P.I.	propidium iodide
PMA	phorbol myristate acetate
SD	standard deviation
SE	standar error
sn.	supernatant
SPF	specific pathogen free
TCGF	T cell growth factor
TCR	T cell receptor for antigen
vacc.	vaccinia
WE-3	viscerotropic starin of LCMV

Contents

Chapter 1

Literature Review

1.1 Role of T cells in immunity

Resistance to infections is based on two lines of defense mechanisms: natural or nonspecific protection and adaptive or specific immunity. Since the natural system does not confer immunity, the term immune response refers primarily to the response of T (thymus derived) and B (bone marrow derived) lymphocytes.

The first sites of natural immunity are the skin and mucosa, which: a) produce chemical disinfectants, b) provide physical defense by rapid turnover of the outer cell layer, c) and in the case of mucosa surfaces contains high level of natural antibodies. If pathogens penetrate these barriers they are confronted by polymorphonuclear (PMN) leukocytes and macrophages which are the hallmark of an acute inflammatory process.

If invasion by the pathogen is so severe that PMN cells and macrophages are unable to contain the infection the third line of defense, adaptive immunity, becomes activated by antigen-presenting cells, mainly macrophages. The antigen-specific immune response is a complex set of reactions involving different types of lymphocytes which mediate their effects both directly and indirectly through the production of soluble mediators. B lymphocytes are responsible for the humoral immune response, producing antigen-specific antibody upon encountering soluble antigen. These antibodies combine with antigen and may: a) promote phagocytosis by acting as opsonins, b) prevent the attachment of microorganisms to cells or mucosa, c) neutralize microbial toxins, d) activate compement-dependent cytolysis, e)activate direct lysis of the microorganism by complement, f) cause agglutination of pathogens, g) render microorganisms nonmotile, h) inhibit the metabolism and growth of pathogens.

T cells generate cell- mediated immunity (CMI) and are present in the lymphoid tissues, especially around the splenic arterioles and paracortical areas of lymph nodes, also in the blood and lymph. Their continual movement through tissues and lymph nodes ensures that antigens or microbes entering the body sooner or later come into contact with T cells. T cells recognise antigen on the surface of cells and different types of T cells carry out different functions, e.g.: a)

release of active substances called lymphokines, b) trigger B cells to make antibody, c) kill host cells carrying the foreign antigen in conjunction with the appropiate histocompatibility antigens.

Although T and B cells encounter different forms of antigen (cell surface bound vs. soluble), they have some characteristics in common: cell surface expression of a clonally distributed antigen receptor molecule, differentiation from a haemopoietic precursors and development of a memory state. Furthermore, they do not operate independently: B cells must receive signals from T cells mediated by growth and differentiation factors or lymphokines.

Thus, the requirement for T cells in both the cellular and humoral immune responses means that they play a central role in the antigen-specific immune response.

1.2 Cell surface molecules

T cells can be identified by the expression of certain cell surface molecules; some of these will be discussed below:

1.2.1 Thy 1

In mice, the Thy 1 antigen was one of the first differentiation antigens to be discovered on thymocytes (Reif and Allen, 1964). It was found to have two allelic forms later designated Thy 1.1 and Thy 1.2. The Thy 1 antigen is coded for by a gene on chromosome nine (Blankenhorn and Douglas, 1972) and is a glycoprotein of 25-30 kd found on thymocytes, peripheral T cells, fibroblasts, epithelial cells and neurons (Reif and Allen, 1964). In contrast to most cell surface proteins that are anchored in the lipid bilayer of the cell membrane by hydrophobic peptide domains, Thy 1 has been shown to be attached to the cell surface via a phosphatidylinositol (PI) linkeage (Low and Kincade, 1985; Malek et al. 1986). The molecule has amino acid sequence homology to immunoglobulin (Williams and Gagnon, 1982) implying that it may function in cell recognition. Some anti-Thy 1 antibodies mediate T cell activation (Gunter et al. 1984; Konaka et al. 1981; Jones, 1983) leading to interleukin 2 (IL-2) receptor expression and IL-2 production.

Cross-linking of murine Thy 1 triggers a rapid rise in the cytoplasmic concentration of free calcium not only in murine T cells but also in Thy 1.2 transfected human cells and murine B-lymphoma cells (Kroczek et al. 1986).

These results suggest that Thy 1 may have a critical role in the activation of T lymphocytes not expressing functional antigen receptors, [(T3/TCR)$^-$ T cells]. In (T3/TCR)$^-$ cell lines monoclonal antibodies against Thy 1.2 can induce a rise in cytoplasmic free calcium, but fail to stimulate IL-2 production. In the absence of co-expressed T3/TCR, stimulation of the Thy-1 molecule results only in increased Ca^{2+} concentration but not IL-2 production (Gunter et al. 1987; Schmitt-Verhulst, 1987). Therefore it is possible that the interaction of Thy-1 on the surface of (T3/TCR)$^-$ cells with its natural ligand in vivo (perhaps in the thymus) leads to an increase in Ca^{2+} concentration and subsequent differentiation events.

1.2.2 TCR associated recognition structures

1.2.2.1 CD3 (T3)

The CD3 antigen of mice, equivalent to the human CD3 molecule, has recently been identified and consists of 25 kd (γ) and 21 kd (δ) glycoproteins and 2 non-glycosylated polypeptides of 26 kd (ε) and 16 kd (ç), the latter forming a 36 kd heterodimer (Samelson et al. 1985a). The molecule is closely associated but non-covalently bonded to the $\alpha\beta$ heterodimer or the γ-chain of the TCR. There seems to be an obligate requirement for the co-expression of CD3 and TCR on the cell surface. (Weiss and Stobo, 1984; Ohashi et al. 1985). CD3 has been identified on the surface of all mature T cells (Bluestone et al. 1987; Brenner et al. 1986), on CD8$^-$/CD4$^-$ TCR $\gamma\delta^+$ early fetal and adult thymocytes (Bank et al. 1986; Lew et al. 1986) and on 'double positive' (CD8$^+$/CD4$^+$) fetal and adult cortical thymocytes (Bluestone et al. 1987). CD3 bearing cells, present early in thymic ontogeny, express functional TCR and, therefore may be important in development of the T cell repertoire (Bluestone et al. 1987; Pardoll et al. 1987).

A monoclonal antibody specific for murine CD3-ε reacts with all mature T cells. The interaction is followed by surface capping of CD3/TCR complexes and by their persistent (48-72h) disappearance (Meuer et al. 1983; Moretta et al. 1987; Pantaleo et al. 1987a), indicating involvement of CD3 in the transduction of activation signals. Antibodies to human CD3 can induce mitogenic responses (Van Wauwe et al. 1980) or inhibit T cell mediated cytolysis (Meuer et al. 1982/a; Leo et al. 1987), but can also induce nonspecific cytotoxic reactivity of CTL (Spits et al. 1985; Mentzer et al. 1985). The latter has been shown to depend on Fc-receptor expression on target cells, which can cause a physical association between T cell and target cell. (Van Seventer et al. 1987).

The δ-chain of the CD3 complex is known to be phosphorylated following T cell activation by antigen. In contrast, both δ and ε are phosphorylated in response to treatment of the cells with phorbol myristate acetate (PMA) (Samelson et al. 1985b). The functional importance of CD3, based on the finding that immune activation of T cells is also associated with downregulation of the surface levels of CD3/TCR complex (Ando et al. 1985; Ledbetter et al. 1986) could be to control the expression and function of the TCR.

The role of the CD3/TCR complex in T cell activation will be discussed in detail later.

1.2.2.2 CD8 (Lyt 2)

Murine CD8 (Lyt 2), equivalent to the human CD8 antigen, is a disulphide bonded glycoprotein of 70 kd composed of heterodimeric units of 2 polypeptide chains, α (38 kd) or α'(35 kd), and β (30 kd). The two forms of the α chain result from alternative splicing of pre-mRNA (Zamoyska et al. 1985).

The α and β chains are encoded by different, linked loci found on chromosome 6, and were originally called Lyt 2 and Lyt 3 (Ledbetter et al. 1981; Walker et al. 1983). The thymic form of CD8 antigen expresses both α and α' subunits , but α' appears to be present at a lower level in peripheral T cells (Walker, 1984). Lyt 2 is expressed on a subset of T cells which are in most cases cytotoxic or suppressor cells (Cantor and Boyse, 1976). The vast majority of $CD8^+$ cells

recognize antigen in association with a class I MHC gene product (rev. by Zinkernagel and Doherty, 1979). The correlation between CD8 expression and class I MHC restriction is far more striking than that between CD8 expression and any other function.

Antisera to CD8 can block the induction and effector phases of alloreactive CTL activity (Nakayama et al. 1979; Hollander et al. 1981; Sarmiento et al. 1980). This inhibition is clonally heterogeneous, i.e. the monoclonal antibody (mAb) concentration required to produce a given degree of inhibition varies markedly between different CTL clones (Glasebrook, et al. 1983). Based on these findings it was proposed that CD8 molecules stabilize the low affinity interaction between T cell receptor and antigen. It was considered therefore that resistance to inhibition by mAb would indicate a high affinity interaction between antigen and its receptor, so inhibition studies with anti-CD8 mAb would reflect the "receptor affinity" of different T cell lines. The theory has been confirmed by Shimonkevitz and colleauges (1985): alloreactive T cell clones which needed high levels of MHC class I expression on target cells for optimal lysis (so probably had low affinity T cell receptors) were more effectively blocked by anti-CD8 mAb than those that required lower amounts of MHC. In contrast, Swain et al. (1984) proposed a model in which the Lyt 2 molecules function by directly binding to monomorphic determinants of the MHC class I molecule on target cells. Transfection of the Lyt 2 gene into $CD8^-$ cells facilitated the interaction of cytotoxic T cells with their targets, although the transfected cell line had lost its original specificity (Dembic et al. 1987). Transfection of T cell receptor α,β genes from a MHC class I-specific, $CD8^+$ T cell clone endowed the host cell with the specificity of the donor cell type only if the same cell had also been transfected with the Lyt 2 gene (Gabert et al. 1987).

So, the view that Lyt 2 molecules are enhancers of receptor avidity is confirmed by these findings and suggests that CD8 molecules may also play a role in transmembrane signal transduction (Emmrich et al. 1986).

1.2.2.3 CD4 (L3T4)

The CD4 molecule is another nonpolymorphic member of the immunoglobulin gene superfamily, expressed on the surface of a functionally distinct population of T lymphocytes. These cells are known as helper - or inducer - T cells, and show specificity for class II MHC molecules on cells (Wilde et al. 1983; Dialynas et al. 1983). The CD4 glycoprotein is also expressed on cells other than T lymphocytes such as bone marrow-derived macrophage-like cells, some B-cells, granulocytes and a shorter transcript on brain parenchymal cells (Shaw et al 1985; Koenig et al. 1986; Maddon et al. 1987; Gorman et al. 1987).

Unlike CD8, which is remarkably different in mouse and human, CD4 has been found as a 55 kd monomer in both species (Dialynas et al. 1983; Terhorst et al. 1980). It has strong evolutionary conservation in its 40 amino-acid cytoplasmic tail (Littman and Gettner, 1987a) suggesting that this region is essential for CD4 function.

Monoclonal antibodies against CD4 have been shown to block numerous T cell functions, such as antigen-specific proliferation, mixed lymphocyte reaction and induction of helper activity (Webb et al. 1979; Wilde, 1983; Swain et al. 1984). The antibodies probably act either by preventing the formation of cell:cell conjugates (Spits et al. 1986) or by transmitting a negative signal (Bank and Chess, 1985; Greenstein and Burakoff, 1987; Tite et al. 1986). As in the CD8 system, the amount of anti-CD4 antibody required to block T cell activation correlates inversely with the amount of antigen required to obtain a particular level of activation (Marrack et al. 1983). The importance of CD4 density has been studied using a murine T cell line expressing human CD4 (Sleckman et al. 1987). This cell line can produce 6-20 fold more IL-2 in response to the appropiate antigen, than the control cell lines. Furthermore, when antigen levels are suboptimal, the response of the cells is entirely CD4 dependent.

While several studies have attempted to confirm that CD4 interacts with MHC class II gene products (Watts et al. 1984; Greenstein et al. 1984 & 1985; Swain, 1984; Gay et al. 1987), some other results suggest that CD4 may not solely be involved in MHC recognition but may have an active role in transmembrane signaling. Anti-CD4 monoclonal antibodies inhibited Con A induced

IL-2 production by a number of antigen-specific T cell hybridomas in an assay system that was free of MHC class II bearing cells (Wassmer et al. 1985). In addition, perturbation of certain epitopes of CD4 molecules by antibodies could inhibit anti-T3 activation even under experimental conditions in which the accessory cells were devoid of MHC class II molecules (Bank and Chess, 1985).

In a similar model, in the absence of class II MHC molecules, Owens and Fazekas de St Groth (1987) have confirmed that CD4 interacts with the T cell receptor during T cell activation. Anti-CD4 antibodies sterically hindered the formation of TCR complexes preventing activation. However, by increasing the epitope density of the activating ligand, the avidity of T cell-ligand interaction could be increased sufficiently to prevent this disruption, which explains the observation that stimuli with superoptimal doses of both soluble or solid-phase coupled F23 (anti-TCR antibody) or with antigen were not inhibited.

Recent studies have also demonstrated that human CD4 is internalized following stimulation with phorbol esters (Hoxie et al. 1986; Solbach, 1982), and that this is accompanied by phosphorylation of a cytoplasmic serine residue (Acres et al. 1986). Parallel downregulation of CD4 and CD3 glycoproteins has been demonstrated (Weyand et al. 1987) following antigenic or PMA stimulations, suggesting that both markers might be members of a multi-molecular complex mediating T cell activation. Further evidence was provided by Saizawa et al. (1987), who showed that the TCR-class II MHC-antigen-CD4 complex is 30-100 fold more effective in T cell activation than cross linking of the T cell receptor complex alone. This experiment also suggests that CD4 is a physical part of the T cell receptor for the antigen - class II MHC complex.

1.2.3 Function associated molecules

1.2.3.1 CD2 and LFA-3

CD2 (also known as T11 or erythrocyte rosette receptor or LFA-2) is a functionally important T lymphocyte surface glycoprotein of a relative molecular mass 45-50 kd on human lymphocytes

(Krensky et al. 1984); a similar molecule probably also exists in mice (Sewell et al. 1987). It is present early in thymocyte ontogeny and on all mature T cells (rev. by Haynes, 1981). Monoclonal antibodies to CD2 inhibit CTL-mediated killing by binding to the T lymphocyte and blocking adhesion to the target cell (Sanchez-Madrid et al. 1982; Krensky et al. 1983 & 1984). Such antibodies also inhibit T helper cell responses including antigen-stimulated proliferation, IL-2 secretion and IL-2 receptor expression (Sanchez-Madrid et al. 1982; Van Wauwe and Goossens, 1981; Palacios and Martinez-Maza, 1982; Krensky et al. 1983; Reed et al. 1985b; Tadmori et al. 1985). Certain combinations of monoclonal antibodies to CD2 epitopes trigger proliferation of peripheral blood T lymphocytes (Meuer et al. 1984), cytotoxic effector function (Siliciano et al. 1985) and expression of IL-2 receptors by lymphocytes (Fox et al. 1985) probably through the phosphoinositide breakdown pathway (Pantaleo et al. 1987b). These results suggest that CD2 can function in signalling as well as being an adhesion molecule. Its natural ligand is probably LFA-3 (lymphocyte function associated antigen-3).

LFA-3 has a molecular mass of 55-70 kd and has a broad tissue distribution including expression on endothelial, epithelial and connective tissue cells in most organs studied and on most blood cells including erythrocytes (Krensky et al. 1986; Selvaraj et al. 1987). MAb to LFA-3, like mAb to CD2, also inhibits a number of T helper dependent functions and inhibits conjugate formation between CTL and target cells (Krensky et al. 1984; Shaw et al. 1986; Mentzer et al. 1987). The binding between CD2 and LFA-3 is of high affinity and can mediate adhesion of lymphoid cells (Selvaraj et al. 1987). CD2 and LFA-3 dependent, antigen independent, CTL-target conjugation does not result in increased cytoplasmic [Ca^{2+}] levels in contrast to the antigen-dependent interaction (Mentzer et al. 1987). Thus, CD2 and LFA-3 appear to act strictly as an avidity enhancing mechanism in this CTL system.

A role in thymic ontogeny has recently been proposed for these molecules. It appears that the adherence of thymocytes to epithelial cells depends largely on the interaction between CD2 and LFA-3. This conclusion is based on the ability of anti-CD2 mAb and anti-LFA-3 mAb to block rosetting of thymic epithelial cells with thymocytes (Singer et al. 1986). Because it is known that the CD2 pathway can be triggered in the absence of the CD3/TCR complex (Moretta et al. 1987),

this interaction might be important in driving proliferation of immature $CD2^+/TCR^-$ thymocytes (Vollger et al. 1987).

1.2.3.2 LFA-1 and ICAM-1

LFA-1 (lymphocyte function associated antigen-1) is a member of a family of molecules, called integrins, found on many tissues of mice (Davignon et al. 1981) and man (Sanchez-Madrid et al. 1982; rev. by Hynes, 1987). Their tissue distribution, structure and function is similar in the two species. LFA-1 is expressed by all leukocytes with the exception of some macrophages. There are 15-40000 LFA-1 surface sites per peripheral blood lymphocyte with more abundant expression on T than B lymphocytes and increased expression on T blasts (Kurzinger et al. 1981). LFA-1 is a heterodimer consisting of an α subunit of 180 kd and a noncovalently associated β subunit of 95 kd (Sanchez-Madrid et al. 1983). Monoclonal antibody to LFA-1 blocks CTL mediated killing by acting at the adhesion stage rather than the lethal hit delivery step (Springer et al. 1982). MAb also inhibits induction of T cell proliferation in response to a wide variety of stimulants (rev. by Dongworth et al. 1985; Springer et al. 1987). The physiological mechanism of activation of LFA dependent adhesion has been hypothesized by Springer et al. (1987) to be that binding of the antigen receptor during the initial contact of T cell with antigen presenting cell or of effector T cell with target cell releases diacylglycerol and stimulates LFA-1 to increase adherence.

The putative LFA-1 ligand, intercellular adhesion molecule-1 (ICAM-1) is widely distributed on cells of both hemopoietic and non-hemopoietic origin. It can be upregulated by IL-1 and IFN-γ, with expression being greatest during inflammation (Dustin et al. 1986; Rothlein et al. 1986). Further studies are needed to clarify whether LFA-1 is the sole ligand or just a member of a family of related ligands. The exact molecular basis of lymphocyte adhesion and signal transduction is also unknown.

1.2.4 Differentiation / activation antigens

1.2.4.1 Activating proteins of the Ly-6 locus

The murine Ly-6 locus controls multiple cell surface molecules with distinct cellular and tissue disribution. Although the function of Ly-6 antigens are unknown, several of these antigens represent markars of T cell differentiation and activation. The original Ly-6 locus was identified by a monoclonal antibody and is now called Ly-6A (Kimura et al. 1980, 1984). Recent studies have demonstrated that Ly-6 encoded antigens play a role in physiological T cell activation: TAP (T cell activating protein) is a 15-17 kd membrane protein which is anchored in the cell membrane via a phosphatidylinositol group (Reiser et al. 1986). Binding of monoclonal antibodies to TAP can result in extensive antigen-independent T cell proliferation (Rock et al. 1986). It is expressed on 10% of mouse thymocytes which are mainly cortisone-resistant and mature. It is also expressed on 70% of peripheral T cells but not on resting B cells (Yeh et al. 1986). All $CD4^+$ cells are TAP^+, but only 50% of $CD8^+$ lymphocytes express detectable amount of TAP. Unstimulated CTL precursors are TAP^-. Upon activation all T and B cell became TAP^+ (Yeh et al. 1986).

During the initial characterization of TAP, a second protein, TAPa was identified (Rock et al. 1986) which is molecularly distinct and coordinately expressed on cells with TAP (Reiser et al. 1986).

Another antigen mapped in the Ly-6 locus having the characteristics of an activation marker is 34.2.11, which has a distinct development and tissue distribution from TAP and TAPa (rev. by Shevach et al. 1986).

Ly-6A.2 antigens appear on T cells after stimulation with mitogenic lectins (Kimura et al. 1984). B4B2 recognizes a subpopulation of approximately 40% of $CD8^+$ T cells which is declines with age (Fichtner et al. 1987).

More recently Shevach et al. (1986) postulated that Ly-6 antigen represents an important alternative pathway for T cell activation, because an anti-Ly-6 monoclonal antibody can act synergistically with phorbol ester as a potent T cell mitogen. These findings were supported by

Havran et al. (1988) who found that anti-Ly-6 mediated stimulation of cloned $CD8^+$ cells cannot be inhibited by anti-CD8 or anti-IL-2 receptor antibodies.

1.2.4.2 Ly 24 (Pgp-1)

The Ly 24 antigen is a 95 kd glycoprotein found on adult mouse thymocytes (Trowbridge et al. 1982). Its functional role is undefined but some evidence suggests that it is associated with the cytoskeleton and may be involved in cell-cell adhesion (Jacobson et al. 1984a and b). The molecule is expressed not only on immature ($CD4^-/CD8^-$) thymocytes (Lesley et al. 1985a and b) but also on some $CD8^+/CD4^-$ and $CD8^-/CD8^+$ 'mature' T cells in the thymus (rev. by Lynch and Ceredig, 1987). Its implications in thymus biology has been reviewed by Lynch and Ceredig (1987). Furthermore its expression appears to distinguish in vivo primed peripheral T cells from unprimed cells (see Chapter 5).

1.2.4.3 J11d

This marker distinguishes differentiation stages of both T and B lymphocytes. It is expressed on most thymocytes but not on peripheral T cells, and on primary but not on memory B cells (Bruce et al. 1981; Symington and Hakomori, 1984). In the thymus it distinguishes functionally incompetent ($J11d^+$) from functionally competent cells ($J11d^-/Lyt2^+/L3T4^-$) (Crispe and Bevan, 1987). $J11d^+$, 'double negative' thymocytes represent probably the main line of T cell differentiation, while $J11d^-$, 'double negatives' have very limited diversification potential in vitro and in vivo and are probably not precursor T cells (Crispe et al. 1987).

1.3. T cell receptor for antigen

1.3.1 Structure

Progress in the search for the T cell antigen/MHC receptor came first in the form of clonotypic antibodies (Meuer et al. 1983; Lancki et al. 1983; Haskins et al. 1983). Evidence that these antibodies recognise T cell receptors was based on their ability to interfere with the binding and antigen-specific effector function of only T cells against which the antibodies were produced. These antibodies could inhibit specific lysis by CTL clones and IL-2 production by T helper clones. They were also capable of activating the T cells when rendered polyvalent by coupling with Sepharose (Meuer et al. 1983).

At the present stage of knowledge there are two types of TCR. Most T cells express a TCR that is a complex comprising a highly variable α and β chain. These are disulphide linked and non-covalently associated with the three monomorphic subunits of a T3 molecule (Meuer et al. 1983; Borst et al. 1983). Another type of receptor complex has recently been identified, comprising a γ-δ heterodimer which is also associated with a T3 molecule (Brenner et al. 1986 & 1987).

In the mouse, the α and β chains have a similar molecular weight (38-44 kd), but in man the two chains are easily distinguishable (α: 44-52 kd, acidic; β: 37-44 kd, basic) (Kappler et al. 1983; Kaye and Janeway, 1984). The loci encoding the mouse α and β TCR chains are located on chromosomes 14 and 6, respectively (Dembic et al. 1985; Lee et al. 1984). Both α and β chain mRNA-s code for a protein with L (hydrophobic leader), V (variable), C (constant), transmembrane and cytoplasmic domains. (rev. by Adkins et al. 1987). The TCR γ chain genomic locus has been mapped to mouse chromosome 13 (Kranz et al. 1985) and is essentially similar in structure to those encoding the α and β chains, although it has significantly less variability (Saito et al. 1984). The polypeptide product has a relative molecular mass 55 kd, and is found with another polypeptide, δ, (40 kd) in association with the T3 glycoprotein on the cell surface (Brenner et al.1986 & 1987). The T3 has a functional role in the $\gamma\delta$ TCR complex, but is structurally distinct in

that there is differential glycosylation of the T3 δ chain on these T lymphocytes (Krangel et al. 1987).

Chien et al. (1987) recently reported the identification of a new T cell receptor gene, thought to encode the δ chain molecule. It preserves many of the characteristics of T cell receptor genes with an extra cysteine residue after the first domain, probably for heterodimer formation, and a conserved lysine residue in the transmembrane region. It has also been sequenced and cloned, the predicted molecular weight of the protein derivative is 37-40 kd (Chien et al. 1987).

It was suggested that there may be an association between antigen specificity of a particular TCR and use of particular Vα and/or Vβ genes. Experiments with a helper and a cytotoxic T cell clone which have completely different antigen and MHC specificity revealed that they both use identical Vα and Vβ segments but different D-J segments for their TCR-s. This suggests that the D and J regions may play a key role in specificity for antigen and MHC (Rupp et al. 1987).

The other type of receptor seems to be present on only about 3% of peripheral $CD3^+/CD8^+/CD4^-$ T cells and on less than 1% of neonatal thymocytes (Lanier et al. 1986) which have $CD3^+/CD8^-/CD4^-$ 'immature' phenotype. They exhibit non-MHC restricted cytotoxicity (Brenner et al. 1987; Borst et al. 1987). Recent studies on the ontogeny of γ expression have revealed a putative role for this receptor (see 1.3.2).

1.3.2 Ontogeny

The ontogeny of α,β and γ gene rearrangement and expression in fetal and adult cells has been studied by several groups. Two major issues have been addressed by these studies. First, do rearrangements occur in a sequence of steps, as is the case of the three immunoglobulin families? Second, when and where in development are these genes first expressed?

The mouse has a gestation period of 20 days and immunocompetent thymocytes can be detected shortly before birth (Widmer et al. 1981). Analysis of transcripts present in the fetal thymus indicates that γ RNA appears first, on day 14 (Haars et al. 1986; Raulet et al. 1985; Snodgrass et al. 1985). At this stage the thymus contains mainly $CD4^-/CD8^-$ cells which express

Thy-1 (van Ewijk et al. 1980) and IL-2 R (Ceredig et al. 1983; Habu, 1984). CD8 is expressed at day 16, closely followed by CD4 (Ceredig, 1983).

DβJβ rearrangements are also detected but transcripts of Vβ genes are present first at day 15 (Haars et al. 1985, Snodgrass et al. 1985). T3 molecules may also be found in these cells and they are present throughout fetal development and in adult thymus (Bluestone et al. 1987). On day 16 a relatively large amount of β gene RNA is present, and α gene transcripts can be detected. On day 17 complete α and β gene transcripts forming $\alpha\beta$ heterodimers appear on the cell surface and the quantity of $\gamma\delta$ gene products is decreasing. The latter cannot be detected by day 20 (Lew et al. 1986; Bluestone et al. 1987; Pardoll et al. 1987).

Experiments on selected populations of adult Lyt2$^-$/L3T4$^-$ (double negative) thymocytes have yielded similar results to those obtained with fetal thymocytes of day 14-16 gestation. About 50% of these thymocytes spontaneously express IL-2 receptor and their anatomical localization is random (Ceredig et al. 1985). These double negative cells have large amounts of γ RNA (Raulet et al. 1985) and very little α RNA (Samelson et al. 1985). A subpopulation of these cells express the cell surface antigen Pgp-1, which is also found on day 14-15 fetal thymocytes (Trowbridge et al. 1982).

The cells having nonproductive γ and/or δ gene rearrangements can further develop and rearrange α/β genes (Brenner et al. 1987), but the cells carrying nonproductively rearranged α and/or β genes probably die in the thymus. There are conflicting estimates of the fraction of cells in this category that express $\alpha\beta$ heterodimers (Royer et al. 1984; Acuto et al. 1985).

The development of a self MHC-restricted repertoire of TCR is also thought to take place in the thymus. According to this thymic education model of T cell differentiation, T cells with particular antigen receptors are selected in the thymus in the absence of antigen (Zinkernagel 1978; Fink and Bevan, 1978). This selection is believed to depend upon MHC molecules expressed by certain thymic cells. Medullary stromal cells express high levels of both H-2K and H-2D antigens, whereas cortical epithelial cells express mainly H-2D allodeterminants (Rouse et al. 1979; Van Ewijk et al. 1980). MHC class II, or Ia,is expressed at a very high levels on cortical epithelium, medullary epithelium and medullary dendritic cells (Rouse et al. 1979; Barclay and Mayrohofer, 1981). Cells expressing Ia antigens are present in medullary regions at 14 days of fetal

development. Class I MHC expression is first detected at day 16, by which time levels of Ia are high throughout the thymus (Jenkinson et al. 1981). Experiments with radiation-induced bone marrow chimeras and thymus reconstituted nude mice, or even with unmanipulated nude mice, demonstrated that the self-MHC restriction specificity of class II-restricted T cells is determined strictly intrathymically (Singer et al. 1982; Kruisbeek et al. 1983) while the specificity of self class I-restricted T cells can not only result from intrathymic interactions (Zinkernagel and Doherty, 1979; Kruisbeek et al. 1981) but also from extrathymic events (Doherty et al. 1981; Ando and Hurme, 1981; Kruisbeek et al. 1983). Furthermore, experiments with neonatal mice, treated with anti-Ia antibody show, that medullary thymic antigen presenting cells (APC) not only determine class II-restriction specificity of $CD4^+$ cells but in fact play a crucial role in the development of the $CD8^-$ /$CD4^+$ lineage (Kruisbeek et al. 1983; Kruisbeek and Longo, 1985).

Despite accumulating results concerning T cell differentiation in the thymus, the exact mechanism of self-tolerance, intrathymic differentiation, and repertoire selection is still not clear.

1.3.3 MHC restricted recognition

1.3.3.1 MHC molecules

T cells recognize antigen on the cell surface only if the appropiate allele of a polymorphic MHC molecule is also expressed. Thus, T cells have a dual specificity, recognizing both antigen and allele specific determinants of the MHC molecule and this recognition of antigen is said to be restricted by the MHC molecule (Zinkernagel & Doherty, 1974a and b).

The murine MHC antigens are members of a large family of closely related genes located in separate, but closely linked regions on chromosome 17. Cell surface MHC gene products can be divided into two classes on the basis of their structure. Class I loci (H-2K, H-2D and H-2L) code for membrane bound glycoproteins with a molecular weight of 44 kd. The glycoprotein molecule is 350 amino acid long, contains three external domains each about 90 residues in length, a

transmembrane region, and a cytoplasmic domain. The third external domain is noncovalently associated with β2-microglobulin (12 kd), a 100 amino acid long polypeptide that shows homology to the constant region domains of immunoglobulins and is not encoded in the MHC (rev. by Hood et al. 1983). Two allelic forms of β2M exist among the common laboratory mouse strains, β2M-A and β2M-B which are represented in Balb and C57Bl mice, respectively (Goding and Walker, 1980). CTL raised across certain H-3 incompatibilities are specific for β2M, suggesting that β2M is a minor H antigen (Rammense et al. 1986).

Sequence studies indicate that the class I heavy chains are among the most polymorphic molecules encoded in the mouse genome. In contrast, β2-microglobulin is relatively invariant. Two alleles of the MHC I-region have been shown to contribute to class II antigens, namely A and E. The products of these loci are often called Ia antigens. Each Ia molecule consists of two noncovalently associated glycoproteins. The heavier chain, known as the α chain, has a Mw of approximately 35 kd and the lighter chain, designated the β chain has a Mw of approximately 28 kd. Unlike the class I antigens, for which only the heavy chain is encoded by genes in the MHC, both chains of the class II antigens appear to be encoded within the I region. The β chains of both I-A and I-E antigens are encoded within the A region, whereas the α chains are encoded in the regions carrying the appropiate letter designations. Furthermore it appears that the polymorphism of α chain is very limited, whereas that of β chain is extensive, suggesting that alloantigenic specificities are predominantly a function of beta chains. Not all H-2 haplotypes express E region antigens. No E antigens have been detected in $H\text{-}2^{b}$, $H\text{-}2^{s}$ and $H\text{-}2^{q}$ (rev. by Klein, 1981).

The tissue distribution of MHC class I and class II antigens is different. The class I antigens encoded by the K and D regions are distributed on all tissues, though some neurons may remain class I MHC negative (Wong et al. 1984a and b), while the tissue distribution of Ia antigens is more restricted. Among peripheral lymphoid tissues, which are the richest source of MHC antigens in general, Ia antigens are expressed predominantly on B cells, dendritic cells and on certain macrophages. The amount of Ia antigen detectable on other tissues is quite small. Whether or not this is of functional significance remains to be determined (rev. by Klein, 1981; Hood, 1983 et al.). It has been shown that expression of MHC class I and class II antigens is

upregulated by interferon-γ and α/β (Steeg et al. 1982; Wong et al. 1984a and b), with γ being much more effective in this regard.

Different subsets of mature T cells are restricted by different classes of MHC molecules. In general, $CD8^+/CD4^-$ cells, usually cytotoxic T cells, recognize antigen in association with class I MHC gene products, whereas $CD4^+/CD8^-$ cells, usually helper T cells recognize antigen in the context of class II gene products (Ia antigens) (Biddison et al. 1978; Meuer et al. 1982b).

1.3.3.2 Antigen presentation

One of the major controversies in immonology in the last few years is the nature of and requirement for processing of antigen for T cell activation. Physiologycally, the antigen presenting cell (APC) may generally use proteolysis, because the processing can be blocked by inhibitors of lysosomal function (Unanue, 1984). However, in some cases unfolding of the protein may be sufficient, and in the case of short peptides or intrinsic membrane proteins, no processing may be required (Streicher et al. 1984; Allen and Unanue, 1984; Walden et al. 1985; rev. by Allen, 1987). In these experiments which used unilamellar or multilamellar lyposomes, the density of antigen could be significantly greater than that present on the surface of APC, making direct comparisons with systems using APC difficult. Different types of APC process and present antigen to different extents, and different antigens require greater or lesser amounts of processing.

There now appears to be strong evidence that processed antigens and MHC molecules can interact with measurable affinities (Babbitt et al. 1985; Buus et al. 1986; Schwartz, 1985; Watts et al. 1986; Allen et al. 1987). Antigen processing can be defined as the biochemical events, during which a protein antigen undergoes structural changes which allow it to associate with an MHC molecule and than to form the determinant recognized by the T cell. According to De Lisi & Berzofsky (1985), the native secondary structure of antigen might not be important, instead the antigen presenting cell may induce and maintain an α helix-like structure of the antigen segment

which provides the antigenic stimuli for TCR. The theory predicts that the antigenic sites have to be amphipathic. The model has been confirmed by some observations (Takano, 1977; Berkower et al. 1985a and b), but other results are not consistent with it. Sette et al. (1987) studied an immunogenic peptide of ovalbumin, consisting of eleven residues and did not find segregation between residues interacting with Ia and TCR, respectively. Their data suggest a two-dimensional planar formation (β-sheet) of the peptide and with this structure each residue could interact with the Ia molecule or with the TCR.

The question of antigen-MHC complex formation in the case of nonresponders is also controversial: affinity binding studies have shown low affinity binding (Kd = 10^{-3} to 10^{-6} M) of an antigenic peptide to high responder but not low responder Ia (Allen et al. 1985/a). However, photoaffinity labelling studies have demonstrated nonspecific binding between antigen and Ia (Abbas et al. 1985; Watanabe et al. 1986).

Work done by Phillips et al. (1986) raises the question of whether the antigen-MHC complexes form extracellularly and/or intracellularly. They have shown that class II molecules located intracellularly bind antigenic peptides better than those located on the cell surface. Perhaps the cell surface class II molecules already have their binding sites occupied by a peptide ligand and those in the golgi and endoplasmic reticulum do not. An MHC molecule may constantly be cycling between the surface and the interior of the cell. Each time it is taken into the cell, it may shed the peptide ligand and pick up another, in which case most of the cell surface located MHC molecules would be 'occupied' and the intracellular, including newly synthesized molecules, would be 'free'. It is not known, where in the cell MHC molecules associate with antigen. It might happen in the lysosome (Buus et al. 1986), in extralysosomal acidic vesicles (Allen, 1987), in the golgi or pre-golgi apparatus (Cresswell, 1985; Phillips et al. 1986).

Protein-gold conjugates showed that the antigen is internalized after 0.5h by adsorptive pinocytosis into endosomes of an antigen-nonspecific APC B cell. (Pernis and Axel, 1985). These conjugates are recycled to the surface membrane in 3-4 h. These kinetics are compatible with the times required to pulse an APC with antigen and for an APC to function in antigen presentation. In the case of antigen, specific APC receptor mediated endocytosis has been proposed (Pernis and Axel, 1985). Their study suggests, that processing and presentation of antigen are no longer the

exclusive capacity of certain cells of the immune system. Any cell which can express MHC class II molecules appears to have the capacity to process and present antigen to helper T cells. (Most cells already express class I molecules and might present antigens in association with these to cytotoxic T cells.) T cells have been shown spontaneously internalize approximately 20-40% of their cell surface class I molecules mainly through coated pits and vesicles, although B lymphocytes have not been shown to internalize class I molecules in this way (Machy et al. 1987).

An important question that remains to be explored is what determines which antigens are seen with class II and which with class I MHC determinants. One possible explanation lies in the time it takes a newly synthesized class I or class II molecule to reach the membrane. The class I transit time seems to be brief, approx. 20 min, while for class II it may take as much as 60-90 min. (Pernis, 1985). Molecules existing at high concentrations intracellularly and in equilibrium with the endocytic system (e.g. endogeneous membrane proteins and viral proteins produced during active infection) may be able to interact efficiently with either the rapidly cycling class I or slower class II molecules. However, exogeneous soluble proteins accumulated sporadically at low concentration may only form a large number of complexes with the slower cycling class II MHC molecules (Germain, 1986; Kourilsky et al. 1987).

The distinction might be based on different trafficing characteristics of vesicles that contain either class I and class II molecules. Vesicles that contain class II might intersect with the endosytic pathway and present peptides from fragmented proteins. Class I-containing vesicles might receive the peptide products from endogeneously synthesized proteins (rev. by Bevan, 1987).

The most important findings on antigen - class I MHC interaction result from crystallography experiments by Bjorkman et al. (1987a and b), who showed that a large 'groove' exist between the α helices of α_1 and α_2 domains. This grove provide a binding site for processed foreign antigens.

1.3.4 Models of T cell recognition of antigen

Two types of models have been proposed to explain the dual specificity of TCR for antigen and MHC product. The first hypothesizes that a single T cell antigen receptor binding site recognizes a combined determinant formed by the restricting MHC molecule and antigen (Zinkernagel and Doherty, 1974a and b; 1978 & 1979; Matzinger, 1981; Schrader et al. 1982). The antigen and the MHC molecule might interact physically with sufficient avidity to form a complex antigen, either before or after encountering the T cell antigen-specific receptor (Schwartz, 1986; Sherman, 1982). It has been shown that a peptide fragment of lysozyme capable of stimulating T helper cells can bind to an appropiate responder (but not the nonresponder) I-A molecule with significant affinity (Babbitt et al. 1985). Furthermore, transfection experiments (Dembic et al. 1986) provided strong evidence that the $\alpha\beta$ heterodimer is critical in the formation of a TCR complex which recognizes both antigen and MHC and that a second receptor is not necessary for MHC restricted recognition. The α and β chain genes from a CTL clone specific for fluorescein (FL) and I-A^d were isolated and transfected into another CTL clone, the specificity of which was against a different hapten and MHC type. The resultant transfectants obtained both the antigen and MHC restriction characteristics of the donor CTL clone.

In recent work Allen et al. (1987) using a 10 amino acid long stimulatory HEL peptide, determined that 3 amino acids comprise the T cell contact residue (epitope) and also that 3 amino acids form the Ia contact residue (agretope). The peptide probably forms an α helix during the interaction with the Ia molecule, before becoming accessible for recognition by the T cell receptor. It also appears, that there is a single binding site for many unrelated peptides on the Ia molecule. Other results suggest that the antigen-specific receptor can bind antigen in the absence of MHC molecules (Siliciano et al. 1985; Rao et al. 1984).

The second model proposes that the T cell receptor, composed of either two separate molecules or two binding sites in the same molecular complex, provides separate binding sites for the antigen and MHC molecule (Parham, 1984; Pernis, 1985; Cleveland and Elanger, 1985). The problems with this model are the following: firstly, independent segregation of antigen and MHC recognition was not found in somatic T cell hybrids expressing two distinct antigen MHC specificities (Kappler et al. 1981). Secondly, when an anti-clonotypic antibody specific for

ovalbumin-reactive hybridomas was used to select particular T cells from a large pool of hybridomas, two hybridomas were found to have the same antigen/MHC specificity plus allo MHC crossreactivity. They used the same α and β chains as that of the original clone to which the antibody had been made (Marrack et al. 1983). This result strongly suggests that the same heterodimer mediates both antigen and MHC recognition. Thirdly, the structure the T cell receptor itself argues against a model in which the α and β chains constitute two independent binding sites (Kronenberg et al. 1986).

The latest crystallographic studies (Bjorkman et al. 1987a and b) presented evidence that a groove between the two α helices of the α_1 and α_2 domains of the human HLA-A2 antigen, located on the top of the molecule is the likely candidate for the binding site for the foreign antigen that is recognised together with HLA by a T cell receptor. The dimensions of the site are consistent with the expectation that class I molecules bind a processed antigen, probably a peptide. These studies also suggest that a peptide is always bound to histocompatibility antigens and its displacement is an essential feature of cellular immunity. The model can be useful to analyse other class I and class II histocompatibility antigens.

1.4 T lymphocyte subsets and their functions

1.4.1 Cytotoxic T cells ($CD8^+/CD4^-$)

The $CD8^+/CD4^-$ phenotype is primarily associated with MHC class I recognition and cytotoxic function (Cantor and Boyse, 1976; Doherty et al. 1976; Sprent, 1978; Meuer et al. 1982b). $CD8^+/CD4^-$ cells appear in the thymus just prior to birth (day 18-20 in mouse) and comprise about 3-8% of total thymocytes (Ceredig et al. 1983; Mathieson and Fowlkes, 1984) and about 25% of peripheral lymphocytes.

Specific activation of resting cytotoxic T cells consists of consecutive steps such as antigen recognition, interleukin 2 receptor (IL-2 R) expression and proliferation leading to the development of fully mature lytic capacity. In a simplified model, proposed by Gromo et al. (1987), the naive, resting CTL are IL-2 R^-, nonproliferating and noncytotoxic. They become IL-$2R^+$ in response to stimulation with antigen, but still do not proliferate and do not have cytotoxic capacity. The conclusion from numerous in vitro experiments, is that these lymphocytes proceed to proliferate only in the presence of IL-2 (Erard et al. 1985a and b; Vohr and Hunig, 1985; Simon et al. 1986; MacDonald and Erard, 1986; Ochoa et al. 1986) or IFN γ (Chen et al. 1986; Gromo et al. 1987) and subsequently become cytotoxic effector cells.

The first step in CTL activation after encountering antigen is the upregulation of IL-2 R expression. Binding studies with isotope labelled IL-2 have revealed that T lymphocytes express two classes of binding site (Robb et al. 1984; Smith and Cantrell, 1985). The high affinity receptor ($Kd = 10^{-11}M$) consists of at least one 75 kd α chain and one 55 kd β chain (Dukovich et al. 1987), probably encoded by separate genes. The α chain has a long cytoplasmic tail. The chains are not covalently associated and both are necessary to form the high affinity binding site (Smith, 1987; Tsudo et al., 1987; Brandhuber et al. 1987). They bind to different, but perhaps overlapping, amino acid residues on the IL-2 molecule (Robb et al. 1987). The chains have also been detected separately on the surface of some cells (Robb and Greene, 1987). The β chain alone (also known as Tac antigen) forms homodimers which can bind two IL-2 molecules with low affinity ($Kd = 10^{-8}M$) (Robb et al. 1981) whereas the α chain alone represents a medium affinity binding site (Kd =

10^{-9}M) and is probably capable by itself of mediating signal transduction and rapid internalization of membrane bound IL-2 (Dukovich et al. 1987; Bich-Thuy et al. 1987; Wang and Smith, 1987). IL-2 itself can induce expression of the β chain but not of the high affinity receptor in resting (G_0) T cells (Smith and Cantrell, 1985; Bismuth et al. 1985). The biological significance of low affinity binding is not known, although it has been suggested to have a negative influence on T cell growth (Kumar et al. 1987). The simultaneous expression of α and β chains seems to be restricted to T cells stimulated via the TCR-antigen complex (Meuer et al. 1984).

The expression of IL-2 receptor after stimulation is transient, being greatest at 3-7 days, followed by a continous decrease (Cantrell and Smith, 1983). After 10-12 days in culture, repeated antigen stimulation can upregulate the expression of high affinity receptors (Robb, 1984; Reske-Kunz et al. 1986). The binding of IL-2 to a high affinity receptor is followed by rapid internalization via coated pits and degradation in lysosomes (Robb et al. 1981; Kumar et al. 1987). Although the nature of the signal transmission is not known, the effect of IL-2 binding is to cause the cell to progress from the late G_1 into the S phase (Robb et al. 1981 & 1984).

The CTL lytic process has been studied since the 1970s. It has three basic stages. These are: a, specific binding to target cells, b, Ca-dependent programming for lysis, c, the 'lethal hit' and killer independent lysis. The initial cell contact is mediated by accessory molecules, such as LFA-1, CD2, LFA-3 and ICAM-1 (Springer et al. 1982; Krensky et al. 1983) but specific recognition via the TCR is necessary for the killing (Lanzavecchia, 1986). Electromicroscope (EM) studies have revealed that membrane bound cytoplasmic granules are present in CTL (Sanderson et al. 1977; Grossi et al. 1983) containing lysosomal enzymes (Masson et al. 1986). It has been shown that cell contact is followed by calcium dependent processes (rev. by Russel, 1983), such as cytoplasmic rearrangement of cell organells (Ryser et al. 1982), interdigitation of cytoplasmic processes between effector and target cell (Barber and Alter, 1978) and secretion of acid phosphatase and serine esterase (Pasternak et al. 1986; Takayama et al. 1987). Simone and Henkert (1980) provided evidence for pore formation in the target membrane at the lethal hit delivery stage, an observation confirmed later by Tschopp et al (1986). Finally, CTL kill targets by triggering an internal disintegration process which causes rapid cleavage of target cell nuclear DNA (Russel, 1983). Killing always occurs in one direction only and CTL once triggered, can also

lyse 'third party' targets (Lanzavecchia, 1986). After killing, CTL are able to kill further targets (Henkart, 1985).

1.4.2 T helper cell ($CD4^+/CD8^-$)

The $CD4^+/CD8^-$ phenotype is associated primarily with MHC class II recognition, helper and DTH function (Cantor and Boyse, 1975; Dialynas et al. 1983). CD4 'single positive' cells appear in thymus just before birth (at day 18-20 in mouse), and represent about 5% of thymic and 15% of peripheral lymphocytes, respectively.

Specific activation of helper T cells requires interaction with accessory cells presenting antigen in the context of MHC class II molecules and also requires the presence of interleukin-1 (IL-1) which is produced by macrophages (Larsson et al. 1980; Williams et al. 1984; Smith et al. 1980). The activated T helper subset is the major source of IL-2 and other lymphokines, such as IFN γ, IL-3, IL-4 and GMCSF (Mosmann et al. 1986a and b; Tite et al. 1985; Mosmann and Coffman, 1987). However it is clear that some Lyt 2^+ cells make IL-2 and other lymphokines. IL-2 was one of the first T cell derived growth factors to be identified (Gillis et al. 1978). It has been identified as a molecule with 15-17 kd molecular weight (Paetkau et al. 1985) and shows structural and functional similarities in different species. (Robb et al 1981). Human IL-2 consists of 133 amino acids arranged in a right-handed, fourfold α helical structure (Cohen et al. 1986) containing a single molecular disulphide bridge, which is necessary for maintainance of bioactivity (Taniguchi et al. 1983; Robb et al. 1984). Although IL-2 is a necessary factor for T cell proliferation, it also activates B cells (rev. by Robb, 1984) and induces IFN-γ secretion by T cells (Farrar et al. 1982; Klein and Bevan, 1983). T helper cells have recently been shown to produce IL-4 (previously BSF-1: B cell stimulatory factor 1) which is a costimulator for proliferation of resting B lymphocytes (Howard et al. 1982) and also stimulates T lymphocytes, immature thymocytes and mast cells to proliferate (Zlotnik et al. 1987; Mosmann et al. 1986a; Widmer and Grabstein, 1987; Trenn et al. 1988). IL-3 is a lymphokine which stimulates growth of mast cells (Tertian et al. 1981; Nabel et al. 1981) and multipotential hemopoietic cells (Ihle et al. 1983). Interferon γ can also be produced by

both $CD8^+$ or $CD4^+$ cells (Kelso et al. 1982). It inhibits viral replication (rev. by Merigan, 1981), enhances macrophage function (Kelso et al. 1982), induces expression of MHC class I and class II antigens (rev. by Wong, 1984a and b) and inhibits the effect of IL-4 on B cells (Mossmann et al. 1986b). Granulocyte-macrophage colony stimulating factor (GMCSF) has been shown to support the growth and differentiation of neutrophil granulocytes and macrophages . It stimulates phagocytosis and antibody dependent killing (ADCC) by T cells and large granular lymphocytes (LGL) (rev. by Shrader et al. 1983)

The T helper subset can be further divided into sub-types by function and spectrum of lymphokines produced (Mosmann and Coffmann, 1987; Lichtman et al. 1987): the first type of helper T cell clone synthesizes IL-2 and IFN-γ (and lymphotoxin) but not IL-4, whereas the second type synthesizes IL-4 but not IL-2 or IFN-γ. Both types of T helper clones synthesize IL-3 and GMCSF.

1.4.3 Cellular interactions during T cell activation

The importance of interactions between different cell types is likely to vary at different stages of the development of the immune response. Presentation of antigen on the surface of APC and possibe interactions between T cell and APC have been discussed above, however the question of T-T help leading to the development of CTL response needs more detailed analysis. The functional distinction between $CD4^+/CD8^-$ helper and $CD8^+/CD4^-$ cytotoxic cells is not rigid and there are numerous exceptions, creating a more complex picture of co-operation between these subsets.

The generally accepted and simplified model, is that IL-2 produced by MHC class II-restricted T helper cells is necessary and also sufficient for differentiation of antigen primed CTL (Nabholz et al. 1978; Smith et al. 1980b; MacDonald and Erard, 1986). However, the results of some other groups indicate that IL-2 alone might not be adequate to support CTL growth and that other factors are also necessary (Wagner and Hardt, 1986; Hardt et al. 1985; Raulet and Bevan, 1982;

Kanagawa, 1983). Furthermore, there is debate concerning whether Ia-restricted T helper cells are the only source of IL-2 necessary for the development of CTL response.

In vivo experiments revealed that vaccinia immune H-2^b effector cells, negatively selected for alloreactivity to H-2^k, can produce strong CTL response to the virus presented in the context of H-$2D^b$ molecules and in the complete absence of MHC class II homology (Ia^k stimulators) (Bennink and Doherty, 1978; Doherty and Bennink, 1979). Similarly, depletion of $CD4^+$ cells did not prevent the development of an effective anti-ectromelia CTL response in vivo, whereas the production of neutralizing antibody was impaired (Buller et al. 1987). These results are comparable with findings in LCMV infected mice (Moskophidis et al. 1987). Experiments with MHC class I incompatible and minor mismached skin grafts have also shown that in vivo depletion of $CD4^+$ cells did not prevent graft rejection and development of cellular cytotoxicity (Woodcock et al. 1986; Qin et al. 1987). Furthermore, nude mice reconstituted with $CD8^+$ cells show rapid, specific rejection of MHC class I disparate skin grafts (Rosenberg et al. 1987). These findings are in accord with the result of Rosenberg et al. (1986) who found that the $CD8^+/CD4^-$ IL-2 producing T cell subset is the critical cell type determining the in vivo rejection rate of MHC class I disparate skin grafts.

Some in vitro experiments also indicate that there may be different subpopulations among CTL (Andrus et al. 1981; Mizuochi et al. 1985; Guimezanes and Schmitt-Verhulst, 1985). Heeg et al. (1987) found a frequency of IL-2 producing $CD8^+$ cells in the class I MHC-restricted response similar to that shown for IL-2 producing $CD4^+$ cells in the class II MHC-restricted response. The vast majority of $CD8^+$ cells segregate into IL-2 producing non-cytotoxic, or IL-2 non-producing, cytotoxic clones. There is a remarkable difference between the kinetics of IL-2 secretion by CD8+ (peaks at day 4, ceases at day 7) and by CD4+ cells (peaks at day 7), respectively.

Taken together, these results do not exclude the possibility and significance of specific T-T collaboration, but suggest that for some antigens the early phase of CTL response is Th - independent this may progress to a later Th - dependent phase. The ratio between the Th - independent and Th - dependent stage could be different from antigen to antigen and may be genetically determined (Sprent and Schaefer, 1985; Sprent et al. 1986a and b).

1.5 Aims of this thesis

The theme of this thesis is to study the requirements of cytotoxic T cell activation and to characterize the CTL response during the anti-viral T cell response.

The early experiments were designed to study the role of IL-2 in the development of a primary anti-viral T cell reponse (Chapter 3). Then, in vitro experiments to optimize the conditions for generating secondary anti-viral CTL response revealed that memory CTL can be re-activated in the absence of added antigen by a combination of phorbol ester and calcium ionophore (PMA and Cal). The specificity of this activity and characteristics of the activated cells at the clonal and subpopulation levels were determined (Chapter 4). This method is used in Chapter 5 to examine the activation requirements of CTL stimulated in the absence of accessory and stimulator cells. The possibility that Ly 24 antigen may be present on memory but not naive T cells is examined in Chapter 5. The phenotypic characterisation of memory T cells was carried out with Felicity Lynch. Ly 24^+ cells from in vivo primed mice were sorted by FACS. This population gave rise to all the specific cytotoxicity following in vitro stimulation.

The experiments in Chapter 6 deal with the specificity and characterictics of the secondary CTL response against a complex antigen consisting of the K^d alloantigen and vaccinia virus.

Chapter 2

Materials and methods

2.1 Animals

Inbred female and male mice (Table 2.1) were bred and maintained under specific pathogen free (S.P.F) conditions at the John Curtin School of Medical Research. The mice were removed from S.P.F facilities at about 6 weeks of age and conventionally maintained until use, generally at 8-15 weeks of age.

2.2 Tissue culture media

2.2.1 RPMI-1640

For maintenance of continuous cell lines, RPMI-1640 medium was used; The powder (Gibco, 430-1800) was dissolved in distilled, deionized water and supplemented with 2g/l $NaHCO_3$ (Gibco, 895-1810), the pH adjusted to 7.2 and the solution filtered through a 0.22 μm GS type Millipore filter, aliquoted into 500 ml bottles and stored at $4^{o}C$. Before use bottles of RPMI were supplemented with 5% foetal calf serum (Flow, 29-101-49) and with antibiotics to give a concentration of 100 i.u. penicillin, 100 μg streptomycin and 50 μg neomycin per ml.

2.2.2 DMEM

Dulbecco's modified Eagle's medium (DMEM) (Gibco, 430-2100) was used for culturing lymphocytes.It was prepared as described for RPMI, but in addition was supplemented with 6mg/l of folic acid, 36mg/l of L-asparagine, 116 mg/l of L-arginine and 2g/l $NaHCO_3$ (Cerottini et al. 1974). Before use 10 mM Hepes (1M solution of N-2-hydroxyethyl piperazine N-ethane sulphonic acid (CSL, Melbourne), $5x10^{-5}$ M 2-mercaptoethanol, 216 mg/l L-glutamine (Gibco, 320-5039), antibiotics and 10% FCS were added. This medium will be referred as MLR medium.

Table 2.1 Genetic composition of mouse strains

strain	haplotype	MHC composition			
		K	A	E	D
C57BL/6J (B6)	H-2^{b}	b	b	b	b
C57BL/10 (B10)	H-2^{b}	b	b	b	b
B10.A(5R)	H-2^{15}	b	b	k	d
B10.A(2R)	H-2^{h2}	k	k	k	d
B10.HTG	H-2^{g}	d	d	d	b
B10.BR	H-2^{k}	k	k	k	k
CBA/J	H-2^{k}	k	k	k	k
Balb/c	H-2^{d}	d	d	d	d
C3H.OH	H-2^{02}	d	d	d	k
(Balb/c x B6)F1	H-2^{db}	db	db	db	db
(CBA x B6)F1	H-2^{kb}	kb	kb	kb	kb
(Balb/c x CBA)F1	H-2^{dk}	dk	dk	dk	dk

2.2.3 Conditioned medium

Lymphocyte cultures were supplemented with a source of IL-2. This was obtained as a supernatant from Con A activated mouse or rat spleen cells ($2x10^7$ cells were pulsed with 5 μg/ml Con A for 2 h and incubated for 18-24 h in serum-free medium) and concentrated on an Amicon PM 10 membrane (Andrus and Lafferty, 1980) or as a supernatant from EL-4 cells stimulated with 10 ng/ml phorbol myristate acetate (PMA, Sigma, St Louis, Mo) for 24-48 hr. PMA was removed by incubation with dextran-coated charcoal tablets (West Chem. DCC 404) (Farrar et al. 1980). Highly purified human recombinant IL-2 was obtained from Amersham (ARN 1010) or Cetus corporation (Emeryville, Ca).

2.3 Maintenance of tumor cell lines

The cell lines listed in Table 2.2 were stored in liquid N_2 in 0.5 ml volumes in RPMI containing 10% FCS and 10% dimethylsulphoxide (DMSO4) (Fluka, 41650). For culture, cells were thawed rapidly at 37 ^{o}C and washed in 10 mls of warm medium and transfered into tissue-culture flasks. The cell lines were maintained in vitro by passaging approximately twice a week. Fibroblasts, grown as monolayers were first trypsinized (0.1% trypsin diluent containing EDTA) for 10 min at 37 ^{o}C to give a single cell suspension, than washed at 300g for 7 min (a Beckman TJ-6 refrigerated centrifuge was used throughout the experiments) and resuspended in fresh medium.

2.4 Screening for mycoplasma

Cultures were checked regularly for mycoplasma contamination by culturing cells for 2-3 days in antibiotic-free medium. Samples of cells were fixed with 3:1 methanol:acetic acid solution and stained with Hoechst-stain (DNA-fluorochrome 33258) and examined for mycoplasma as shown by cytoplasmic fluorescence using a UV-microscope.

Table 2.2 Tumor cell lines

cell line	origin	cell type	MHC composition
L929	mouse	fibroblast	$H\text{-}2^{kk}$
MC57	mouse	fibroblast	$H\text{-}2^{bb}$
P815	mouse	mastocytoma	$H\text{-}2^{dd}$
EL-4	mouse	thymoma	$H\text{-}2^{bb}$
YAC-1	mouse	lymphoma	$H\text{-}2^{dk}$
KD2SV	mouse	fibroblast	$H\text{-}2^{dd}$
KHTG.SV	mouse	fibroblast	$H\text{-}2^{db}$
K5R.SV	mouse	fibroblast	$H\text{-}2^{bd}$
VERO	monkey	fibroblast	
143 B	human	TK^- fibroblast	
BHK	hamster	kidney	

2.5 Viruses

2.5.1 Preparation of virus stocks

Lymphocytic choriomeningitis virus (LCMV).

The Armstrong E350 (neurotropic) and WE-3 (viscerotropic) strains of LCMV virus were originally obtained from Dr F. Lehmann-Grube (Univ. of Hamburg, Germany) and have been maintained at the John Curtin School of Medical Research since 1974. WE-3 strain grown in L929 cells was used to infect cells in vitro. Confluent L929 cells monolayers were washed with phosphate-buffered saline (PBS pH 7.4)) and infected with 1 ml of virus suspension at a m.o.i. (multiplicity of infection) of 2-3. The flasks were incubated for 1 hour at 37^{o}C and then incubated in 50 ml of RPMI/ 2% FCS until approximately 80% of the cells were destroyed by the virus. The flasks were than stored at -70^{o}C. After thawing, cells and supernatant were centifuged at 1000g for 10 minutes. The pellet was resuspended in about 10 mls of the supernatant and cells were sonicated for 3x20 sec at 4^{o}C. Cell debri was pelleted at 1000 g for 10 min at 4½C. Supernatant was aliquoted in 200 μl volumes and stored at -70 ^{o}C.

The WE-3 strain for immunization was propagated in BHK cells and virus stocks were prepared in a similar fashion to L929-passaged virus. BHK-passaged WE-3 virus was administered intravenously (i.v.) at 1000 LD_{50} per mouse unless otherwise stated.

Armstrong strain of LCM virus was grown in suckling mouse brain, prepared as a 10% homogenate and titrated i.c. as described in section 2.5.2.

Vaccinia virus

Vaccinia virus WR strain was propagated in L929 monolayers in roller bottles containing 100 ml medium. Otherwise the procedures were similar to those described above. Cells were sonicated in 15 ml of supernatant. Titration was performed on L929 cells as described below (section 2.5.2).

Recombinant (VV-H2K^d-6 and VV-TK$^-$) vaccinia viruses

The VV-H2K^d-6 virus strain was prepared by and obtained from Drs Coupar, Boyle and Andrew (CSIRO,Animal Health Laboratory,Geelong, Victoria). A recombinant plasmid vector containing an oligonucleotide adaptor at the 5' end of the coding sequence of the H-2K^d cDNA and also the vaccinia virus promoter was used to transfect TK$^-$ cells infected with vaccinia virus WR strain. Homologous recombination between the vaccinia virus flanking sequences in the plasmid and the thymidine kinase (TK) gene of the infecting virus resulted in a TK$^-$ recombinant virus (VV H2K^d-6) with the chimeric promoter H-2K^d sequence interrupting the vaccinia virus TK gene. Recombinants were selected in the presence of 5-bromodeoxyuridine (Coupar et al. 1986). The TK$^-$ vaccinia virus WR strain was originally provided by B. Moss (National Institutes of Health, Bethesda, MD). The virus strains were propagated in CV_1 and titrated in the 143B TK$^-$ human cell line, basically as described for the vaccinia virus WR strain.

On each occasion virus from a fresh vial was used and thawed rapidly at 37^{o}C just before use.

2.5.2 Titration

Virus stocks were titrated on L929 monolayers, grow in 24 or 6 well trays. The monolayers were washed 3x with PBS. Virus preparation was serially diluted 1:5 in serum-free medium from 10^{-3} and 100 μl volumes were added to four wells for each dilution. The plates were incubated with frequent shaking for 1 hour at 37 ^{o}C, then the fluid was removed by Pasteur pipette and a mixture of 1 ml of 1.5% agar and 1 ml of 2x concentrated RPMI/2% FCS/PSN was added to each well. Plaques were counted 48-72 h later under light microscope (Olympus, Japan) after staining the monolayer with 1% methylene blue containing 10 % formalin.

The titers of BHK-passaged WE-3 virus and in vivo passaged Arm viruses were determined in vivo by intracerebral injection into of serial 10-fold dilutions of virus into groups of adult mice and the titre expressed as the 50% lethal dose (LD_{50}), as calculated by the Kaerber equation:

$\log LD_{50} = 0.5 + \log \max c^a - (\text{sum \% of dead animals} : 100)$

where a) is the logarithm of the highest concentration of the virus used.

2.6 Preparation of cell suspensions

For removing lymphoid organs, mice were killed by cervical dislocation, whereas for collecting cells of cerebrospinal fluid (CSF) the animals were anaesthetized with avertin (2-methyl-2-butanol, containing 1g 2,2,2 tri-bromoethanol, Fluka, Switzerland) and exsanguinated.

The viable cell number of single cell suspensions was determined by the trypan blue dye exclusion method using a Neubauer haemocitometer.

2.6.1 Spleen and lymph node cells

Spleens or lymph nodes (inguinal and axillary) were removed aseptically into ice-cold DMEM, then fragmented with scissors and gently squeezed through a stainless steel sieve The cell suspension was then thoroughly aspirated by Pasteur-pipette and large clumps removed by rapid filtration through nylon gauze. After washing at 300g for 7 min at $4^{o}C$ cells were resuspended and kept at $4^{o}C$ until use.

2.6.2 Thymus cells

To avoid contamination by parathymic lymph nodes, only parts of thymic tissue far from the isthmus were removed. Single cell suspension was prepared as described for spleen cells.

2.6.3 Peritoneal cells

Unstimulated or thyoglycollate-induced (3ml of 3% w/v thyoglycollate i.p., 3-4 days before cell collection) peritoneal cells were collected by washing the peritoneal cavity with 3 mls of ice-cold Hank's balanced salt solution (HBSS). The suspension was removed aseptically through a syringe without opening the interior abdominal wall.

2.6.4 CSF cells

Samples of CSF were collected from the cisterna magna by the method of Carp et al. (1971) adapted by Doherty (1973). The dura mater covering the cisterna magna was exposed by removing the skin and muscles from the atlanto-occipital region. After perforating the membrane with a 26 gauge needle, the CSF was aspirated with a 20 μl pipette (Microcaps, Drumond, Philadelphia). CSF samples were diluted in 0.1% trypan blue and counted in a hemocytometer.

2.7 Separation of lymphoid cell populations

2.7.1 Ficoll-Hypaque density gradient centrifugation

Ficoll-Hypaque (l.08 g/ml) was used to remove cell debris and red blood cells from cell suspensions, (Davidson and Parish 1975). This was prepared by mixing 12 parts of 14% Ficoll 400 (Pharmacia 17-0400-01) with 5 parts of hypaque (32.8 % metrizoic acid, Sigma, m-4762). Cells in 5 mls (up to $2x10^8$) were layered onto 4mls of Ficoll-Hypaque and centrifuged at 3000 g for 20 mins at 22^{o}C. Cells at the interface were collected by Pasteur pipette, resuspended in MLR medium and washed.

2.7.2 Nylon wool column

Lymphocytes were separated on nylon wool columns as described by Julius et al.(1973). Briefly, 1-1.5x 10^8 cells were loaded onto a nylon wool column, allowed to adhere for 45 mins at 37 ^{o}C, then nonadherent cells were recovered by washing the column with 20 mls of warm medium. This protocol resulted in a population of spleen cells containing approximately 70% T cells, as checked by FMF.

2.7.3 Treatment with antibody and complement

Monoclonal antibodies used during the experiments are summarized in Table 2.3. Antibodies were obtained by collecting the supernatants from hybridoma cells growing in MLR medium. For depletion of cell populations single cell suspensions at a concentration of $5x10^6$- $2x10^7$ cells/ml in serum-free medium were incubated with the optimal amount of antibody at 37 ^oC for 20 mins. Low cytotoxicity rabbit complement prepared by the agarose absorption technique (Cohen and Schlesinger, 1970) of serum from selected rabbits was then added at 1/24-1/30 and the cells incubated for a further 45-60 minutes. Cells were then washed and the effectivity of depletion checked by FMF. The optimum concentrations of antibody preparations were determined by incubation of dilutions (serial 2-fold) of antibody with normal thymocytes followed by treatment with complement.

2.8 In vitro stimulation of the secondary immune response

2.8.1 Specific stimulation with antigen

For antigen-specific restimulation of in vivo primed virus-immune lymphocytes, thyoglycollate-induced peritoneal macrophages or spleen cells were used as antigen presenting cells. The cells were depleted with anti-Thy.1 monoclonal antibody and complement as described above (section 2.7.3). They were infected with vaccinia virus for 1 hour at a multiplicity of infection (m.o.i.) = 2.0, or with LCMV WE-3 for 48 hours at a m.o.i. of 0.1. Peritoneal cells were dispensed at a concentration of $5x10^5$ per well in l ml volumes in 24 well trays (Linbro, 7606305). The cells were allowed to adhere for 1-4 hours then the supernates were removed and the virus was inactivated by irradiating the wells with 1200 μW/cm2 UV light for 10 minutes (7200 J/m2) using a 30 W Phillips germicidal lamp. Spleen cells were dispensed in 0.1 ml volume/well and irradiated. Responder cells were then added at a 10:1 ratio ($5x10^6$/well) and the cultures were supplemented with 10% EL-4 supernatant unless otherwise stated. Cells were harvested after 3-5 days, counted and their cytotoxic activity determined in a 6 hr ^{51}Cr-release assay (see section 13.1). In some experiments virus infected stimulator cells were irradiated with γ-ray (2000 rad, ^{60}Co-source).

Table 2.3 Monoclonal antibodies

mAb	origin	subclass	specificity	reference
GK1.5	rat	IgG^{2b}	L3T4a	Dialynas,1983
AT83A	rat	IgM	Thy1.2	Sarmiento,1980
RL 172	rat	IgM	L3T4	Ceredig,1985
3.168.8	rat	IgM	Lyt2	Sarmiento,1980
J11d	rat	IgM	B cells, thymocytes	Bruce, 1981

2.8.2 Stimulation with PMA and Cal

Cells were cultured at 0.5-1.0x10^6/ml in 24 well trays or tissue culture flasks. They were stimulated with 1 ng/ml phorbol myristate acetate (PMA), (Sigma, St.Louis, Mo). and 100 ng/ml of calcium ionophore (Cal),(Ionomycin, Calbiochem, Behring Diagnostics, La Jolla, Ca). These reagents were stored at -70^{o}C as stock solutions of 10 μg/ml (PMA) and 100 μg/ml (Cal) in absolute ethanol. The cultures were routinely supplemented with 2% EL-4 sn and cultured for 3-4 days.

2.9 Limiting dilution analysis of CTL precursor frequency

2.9.1 Specific stimulation

Antigen presenting cells were obtained and processed as described in section 8.1 and dispensed at 10^5 cells/well in 96 well U bottomed microtiter plates (Linbro, Flow, Va 76-042-05). Graded numbers of responder lymphocytes were added into 16 wells for each responder cell dilution. Each culture was supplemented with 10% v/v EL-4 sn, added on days 0, 2, and 5. The cultures were incubated for 8 days at 37 ^{o}C and the cytotoxic activity of cells in each well was determined after resuspending and dividing each culture in half. Virus- infected and uninfected ^{51}Cr-labelled target cells were added at a concentration of 5x10^3 in 100 μl/well and the assays incubated for 6-8 hours at 37^{0}C. Cultures were considered as positive for CTL activity when ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release from targets incubated with stimulators alone by at least 3 standard deviations, or if this was less than 5% lysis, the latter was chosen as the cut-off point.

2.9.2 Stimulation with PMA and Cal

Graded numbers of nylon-wool enriched lymphocytes were cultured in 1ng/ml PMA and 100ng/ml Cal in 96 well U-bottom microtiter trays at 200μl/well for 7-8 days. EL-4 sn (10% v/v)

was added at the initiation of incubation with a further 5% v/v being added every 48 hours. The contents of individual wells were divided in two at the end of the culture period and assayed as described in 2.9.1. A level of 5% specific ^{51}Cr release was considered to be positive.

2.10 Clonal assay of CTL specificity

Effector cells were cultured in 96 well U-bottom trays for 8-10 days. The initial cell number was chosen to be at or near to clonal level (i.e. probability of clonality was >80%) Cells were stimulated with PMA and Cal and supplemented with EL-4 sn as described for LDA (2.9.2). The contents of individual wells were divided in two at the end of the culture period and assayed for cytotoxic activity on different target cells as described. For each group 16-48 individual wells were tested.

2.11 Proliferation assay.

2.11.1 [3H]-thymidine uptake

Cells stimulated either with antigen or mitogen were cultured at 10^5 cells/well in microtiter trays for 2-3 days. Each well was pulsed with 1 μCi [3H]-thymidine (New England Nuclear, Boston, Mass.) for 6 hr. The cells were harvested onto glass fibre filter paper using a PhD cell harvester. Scintillation fluid (1 ml) was added into each scintillation vial (Bunzl, 505) and [3H]-thymidine uptake was measured using a Packard scintillation counter and expressed as cpm.

2.11.2 Colorimetric MTT (tetrazolium) assay

In some experiments tetrazolium salt has been used to measure proliferation as described by Mosmann (1983). MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove any insoluble residue of MTT. At the end of the culture period 10μl stock MTT solution was added into the wells

containing cells in 100 μl volumes and the plates were incubated at 37 ^{o}C for 4 h. Acid-isopropanol (100 μl of 0.04N HCl in isopropanol) was added to all wells and resuspended until the crystals dissolved. Plates were read within an hour on a MR 600 Microelisa plate reader (Dynatech) using a test wavelength of 570nm, a reference wavelength of 630 nm and a calibration setting of 1.99 or 1.00 if the samples were strongly coloured.

2.12 Assay for IL 2 production

Serial, two-fold dilutions of IL 2 containing supernatant from stimulated cells (50 μl/well) were added to 1-5x10^{4} IL 2- dependent CTLL-2 cells (Gillis et al. 1978). Dilutions of an IL-2 containing supernatant was used as a positive control. Cells were incubated for 24 hours and their proliferation measured as described (section 2.11). Cells were also incubated in medium alone to check that they were IL-2 dependent.

2.13 Assay of cytotoxic activity

The cytotoxic activity of immune spleen cells, or in vitro restimulated cells was determined using virus-infected or uninfected continuous cell lines or thioglycollate- induced peritoneal macrophages. In all experiments target cells were preincubated for 48 hours with LCM virus and for 1-4 hours with vaccinia virus, respectively.

2.13.1 ^{51}Cr release assay

Target cells were labelled with ^{51}Cr as originally described by Brunner et al.(1968). The isotope was obtained as a $Na^{51}CrO_4$ solution (Amersham, England.) Target cells at 10^{7} cells/ml were incubated for an hour at 37 ^{o}C with 100 μCi/ml $Na^{51}CrO_4$. then washed 3 times (300g, 7 min). Target cells at 5x10^{4}/ml, (5000 cells/well) were dispensed into 96 well U-bottomed trays. Two-fold diluted effector cells were then added in triplicate and incubated for 6-8 hr at 37^{o}C prior to taking 100 μl of supernate from each well for assay of ^{51}Cr-release.

In the case of macrophage targets, thioglycollate-induced peritoneal macrophages were plated at $8x10^4$ cells/well, (Müllbacher and Blanden, 1979). After they had adhered each well was washed 2x with 150μl serum-free medium, finally about 1 μCi $Na^{51}CrO_4$ was added in 50 μl to each well. Trays were incubated for an hour at 37^{o}C, washed twice with 150μl MLR medium/well, incubated for a further 1-2 hr and washed twice again. The medium was then replaced with 200 μl MLR media containing diluted effector cells. The assay was harvested as described above.

The minimum and the total ^{51}Cr release were determined by incubating the target cells in medium without adding effector cells and adding 100 μl 0.2% Triton-x/well, respectively. Results are expressed as % specific ^{51}Cr release, calculated as:

$$\frac{\text{\% release induced by effector cells - \% spontaneous release}}{\text{\% total release - \% spontaneous release}} \times 100$$

2.13.2 Colorimetric assay.

In the colorimetric cytotoxic assay (Parish and Mullbacher, 1983) $8x10^4$ thioglicollate-induced peritoneal macrophages in 200 μl DMEM containing 1% FCS were plated into 96 well U-bottom trays. After 1hr incubation at 37^{o}C, 100 μl of serially diluted (two-fold) effectors were added in triplicate. Trays were incubated for 6 hr then the supernates were replaced with 100 μl warm 0.036% neutral red in HBSS (Hank's balanced salt solution)/well. After 20 mins incubation at 37^{o}C plates were washed twice with PBS and neutral red was released from live cells with 200 μl 0.1N acetic acid + 0.5% SDS solution/well. After mixing the optical density (OD) of each well was measured by a Microelisa plate-reader. The machine was blanked on wells that were stained with neutral red but did not contain any macrophages, and control wells consisted of macrophage targets which were cultured with medium alone. Results were expressed as percent lysis of targets at each effector:target ratio based on the formula:

$$\text{\% lysis} = \frac{C-T}{C} \times 100$$

where C = OD of control wells, T = OD of wells containing T cytotoxic cells.

2.14 FMF analysis

2.14.1 Phenotypic analysis

For indirect staining of lymphoid cells the antibodies and fluorochrome conjugates detailed in Table 2.4 were used. The antibodies marked with (*) were also used in a purified, biotinylated form, obtained by the method described by Heitzmann and Richards (1974). Both first and second step reagents were titrated for optimal staining before use.

Lymphocyte populations for analysis by FACS were separated on Ficoll before staining, to give as high a concentration of viable cells as possible. Although dead and red blood cells can be gated out,this is less efficient if the proportion of live cells is low.

Aliquots (10^6) of lymphoid cells were incubated for 20 minutes at 4^oC in 100 μl of monoclonal antibody then centrifuged (300g, 10 min) through an underlay of 0.5 ml cold FCS. The pellet was resuspended in 100 μl of fluorescein conjugate. Control samples were stained with the second step reagent only. Following a further 20 mins incubation at 4^oC and washing, cells were resuspended in 0.5 ml medium for FMF analysis.

In the case of F23 and 2C11 antibodies, cells were first incubated for 20 mins with antibody at 37^oC in the presence of 100 μl 0.02% Na-azide according to the method of Leo et al. (1987).

For the determination of DNA content cells were stained with 50μg/ml of the DNA binding dye propidium iodide (PI) in the presence of 0.05% Triton X-100. Cells were analysed immediately by flow cytometry or fixed if analysis was to be done at a later date. Only samples containing less than 5% dead cells were fixed to avoid false positive results. For fixations cells were resuspended in 0.5 ml PBS after the last wash and the same volume of paraformaldehyde (2 %) solution (Sigma, St.Louis, Mo) was added. Samples were kept at 4^oC and run within the next few days on a Becton Dickinson FACS 440 microfluorometer (Mountain View, Ca) linked to a Consort 40 computer. In general, 20000 gated events were collected for each sample. The data were collected and presented as the size and fluorescence of cell populations in the form of one dimensional histograms in which the horizontal axis shows units of fluorescence or light scatter

Table 2.4 Antibodies and fluorochrome conjugates for FMF

mAb	origin	subclass	specificity	reference
AT83*	rat	IgM	Thy1.2	Sarmiento,1980
53.6.7*	rat	IgG_{2a}	Lyt.2	Ledbetter,1979
PC61*	rat	IgG	IL-2R	Ceredig,1985
H-129*	rat	IgG_{2a}	L3T4	Pierres,1984
C71*	rat	IgM	Pgp1.2	Lesley, 1982
RAM*	rat	IgM	Pgp1.1	Lesley, 1982
1M7*	rat	IgG_{2b}	Pgp1	Trowbridge,1982
Mel 14	rat	IgG_{2a}	homing rec.	Gallatin, 1983
F23	mouse	IgG_{2a}	TCR $V\beta_{8,1,2,3}$	Staerz,1985
KJ16	rat		TCR $V\beta_{8.1.2}$	Haskins,1984
2C11*	hamster		T3-ε	Leo, 1987

Fluorochrome conjugates	Obtained from	dilution
Sheep anti-rat Ig-FITC	Silenus, Melbourne	1/20
Sheep anti-mouse Ig-FITC	Silenus, Melbourne	1/10
Sheep anti-hamster	Jackson,Imm.Res.Prod.	1/20
FITC conjugated avidin	Tago, Inc.,Burlingame	1/30

(size) per cell and the vertical axis shows frequency of cells. The percentage of stained cells was calculated by subtracting the value for control cells incubated with second step reagent alone.

14.2 Cell sorting

Staining of cells for sorting on FACS was performed as described above. Cells positive or negative for that specific antibody were collected at the same time into sterile tubes under sterile sorting conditions. Small samples of the sorted populations were re-run to check the effectiveness of the separation. The extent of contamination (frequency of undesired cells) was usually <5%.

2.15 Statistical evaluation of data

Minimal estimates of CTL precursor frequency according to the Poisson distribution were obtained as the slope of the line relating to the number of responder cells per well (plotted on linear x axis) and the percentage of non-responder cultures (plotted on logarithmic y axis) fitting the data by the least squares method (Taswell et al. 1981; Ryser and MacDonald, 1979). The data were analysed on a Texas 74 calculator, the frequency of precursors and the correlation coefficient is given. Linear regression analysis was also calculated using the inbuilt programs of the Ti-74 calculator.

Chapter 3

Requirements for the development of a primary anti-viral cytotoxic T cell response <u>in vivo</u>.

3.1 Introduction

Recovery from poxvirus infection depends essentially on the cell-mediated immune (CMI) response initiated by class I MHC-restricted T lymphocytes (Blanden et al. 1975). When cells are infected with poxviruses, viral antigens appear on the surface of infected cells and they then become susceptible to immune lysis. Effector cells which kill mouse pox (ectromelia) virus-infected target cells in vitro, and trigger virus clearance in vivo, are detectable by day 4 and reach peak activity in the spleen by days 5-6 (Blanden and Gardner, 1976).

In this chapter, the requirements for the development of an in vivo anti-vaccinia CMI response are described based on observations using an immunosuppressed model. This model involved pretreatment of mice with a high dose of cyclophosphamide (CY) which converts the subclinical infection with vaccinia virus into a lethal disease. The clinical outcome of infection parallels with impairement of the CTL response measured in vitro. The possible roles of different factors necessary for the development of the T cell response are studied.

CY has found extensive use as a cytotoxic agent in tumor therapy and for manipulation of the immune response in both clinical and experimental situations (Goodman and Gilman, 1975; Anon, 1981; Turk and Parker, 1982). This short-acting drug is activated in the liver microsomal system to 4-hydroxycyclophosphamide, which in turn gives rise to the cytotoxic species phosphoramide mustard and acrolein: the former binds nucleic acid, preferentially guanine, while the latter binds to protein (Anon, 1981; Hemminki, 1985). The effect of CY on the immune system depends on the amount of drug given and the time of administration relative to antigen stimulation. Proliferating cells are much more susceptible (Goodman and Gilman, 1975; Anon, 1981; Turk and Parker, 1982), so quite small doses are highly immunosuppressive if administered after antigen (Allan and Doherty, 1985). Injection of mice with larger amounts of CY (200-300 mg/kg) prior to antigen kills more than 80% of spleen cells (Taswell et al. 1979; Doherty and Allan, 1984) with different mouse strains showing varying levels of susceptibility (Pevnitsky et al. 1985). The remaining lymphocytes may, however, be able to mediate some aspects of the immune response (Doherty and Allan, 1984). In general, T cells are slightly less susceptible than B cells (Turk and Parker, 1982; Wilmer et al. 1984),

but different types of lytic function mediated by cytotoxic T cells (CTL), natural killer cells (NK) and lymphokine activated killer cells (LAK) are all suppressed for a time following administration of a large dose of CY (Taswell et al. 1979; Doherty and Allan, 1984; Hurme et al. 1982; Ballas, 1986; Allan and Doherty, 1986).

As demonstrated below, the lethal disease that is induced by high doses of CY is associated with both a massive decrease in the total number of effector cells available to combat the infection and with heritable changes in the properties of the surviving T lymphocytes.

This model proved useful for analysing the importance of T cell proliferation, interleukin 2 production and expression of T cell antigens during the development of an anti-viral T cell response in vivo.

3.2 Results

3.2.1 Consequences of CY pretreatment

Administration of 300 mg/kg of CY to B6 mice prior to infection with vaccinia virus (following the protocol shown in Fig 3.1) induces a disease that is lethal within 10 days (Fig. 3.2). This is accompanied by a dramatic drop in the number of spleen cells obtained at different times after the treatment with the drug (Fig. 3.3). However, by day 8 after immunosuppression, the number of spleen cells from non-immune animals exceeds those of the virus-immune and untreated animals, showing that the recovery after immunosuppression is greatly inhibited in the virus infected-animals.

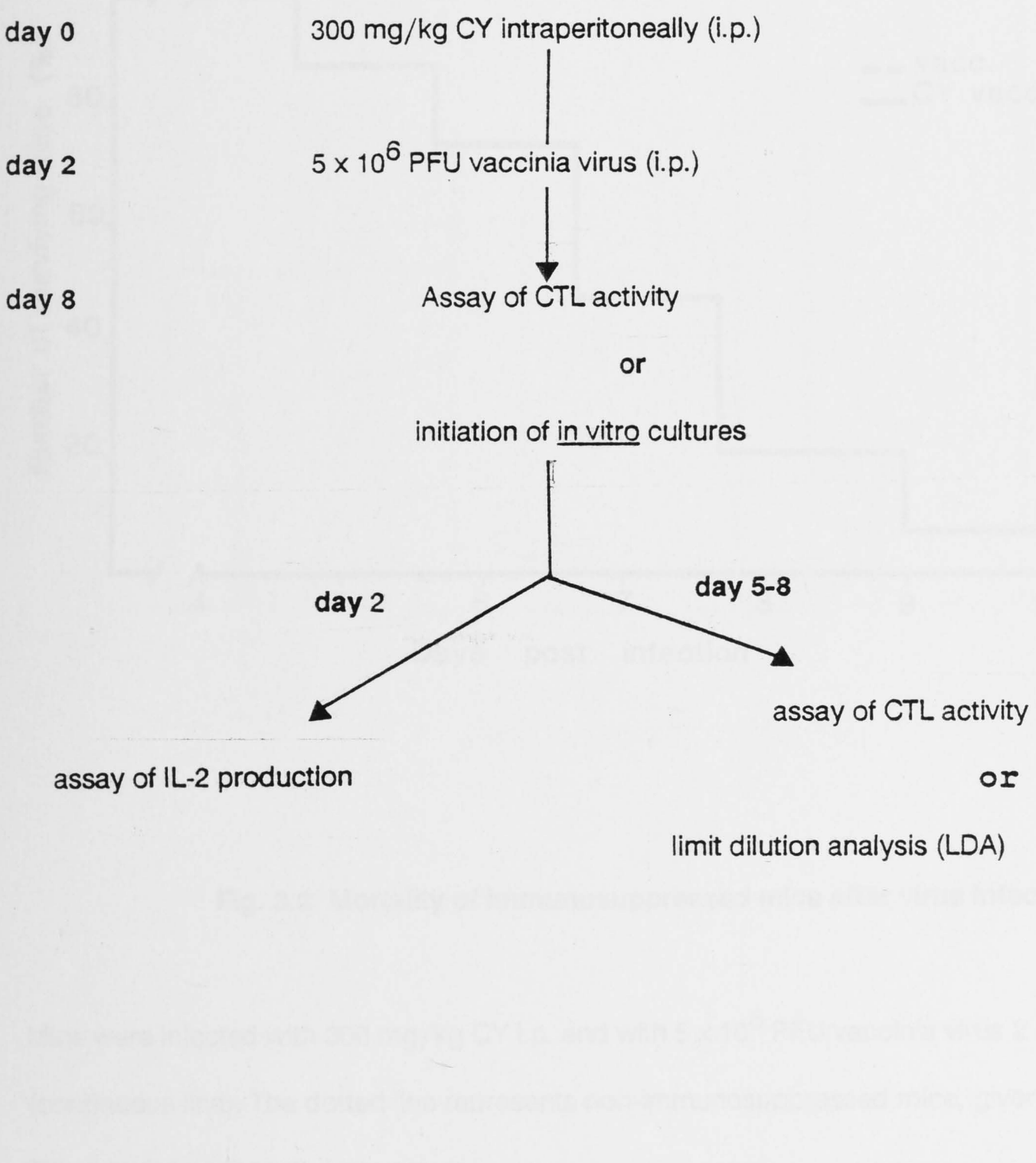
3.2.2 Cytotoxic activity of acute virus-immune spleen cells

The CTL response in ectromelia and in vaccinia infection, peaks at about day 6 after infection (Blanden et al. 1976). CY pretreatment greatly diminishes this activity on a cell-for-cell basis (Table 3.1). Lysis of the NK sensitive YAC target cells, which normally peaks at day 4 after infection, is also generally low in immunosuppressed mice: the slight increase observed here on day 6 might reflect a delay in this activity (Table 3.1, last column). Lower doses of CY (200 or 100 mg/kg) have a less marked immunosuppressive effect on anti-viral cytotoxicity (Fig. 3.4).

3.2.3 Frequency of vaccinia-immune precursor CTL

Virus-immune spleen cells cultured under limit dilution conditions (see Chapter 2, section 9), have a vaccinia-specific precursor CTL (pCTL) frequency of about 1/1000 - 1/1800. Unlike the marked loss of CTL activity, there is only a minor decrease in pCTL frequency following suppression with CY (Figs.3.5 & 3.6). In three separate experiments the decrease was 1.5, 2.6 and 1.3-fold (Table 3.2).When the results of a representative experiment are calculated to express primary cytotoxicity as lytic units (LU) per spleen, and the number of pCTL is also given on a per spleen basis, the discrepancy between acute effector function and the size of the potential pool of responders

Fig. 3.1 Experimental protocol



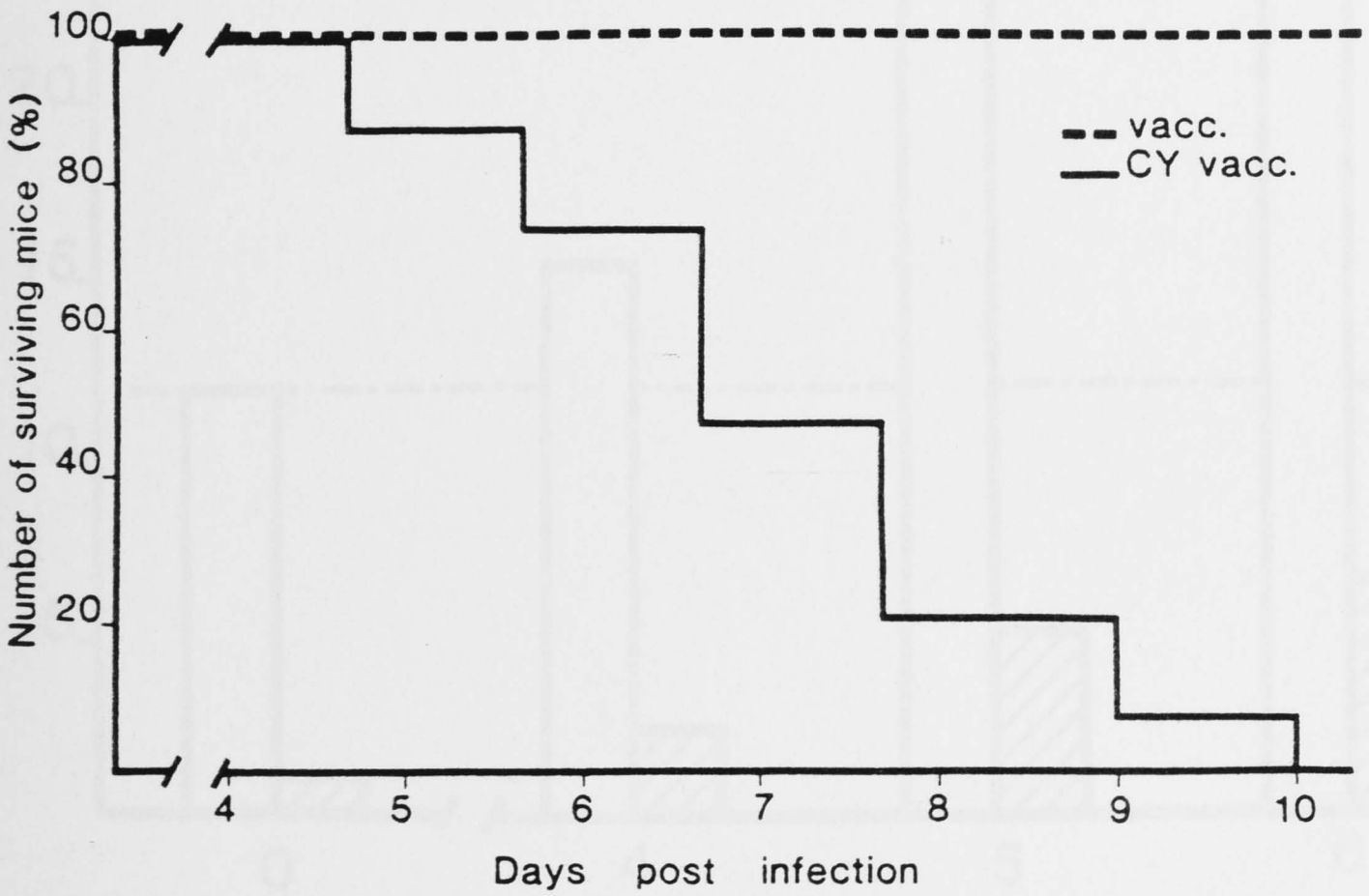


Fig. 3.2 Mortality of immunosuppressed mice after virus infection

Mice were injected with 300 mg/kg CY i.p. and with 5×10^6 PFU vaccinia virus 2 days later (continuous line). The dotted line represents non-immunosuppressed mice, given the virus only. Fifteen mice were tested per group.

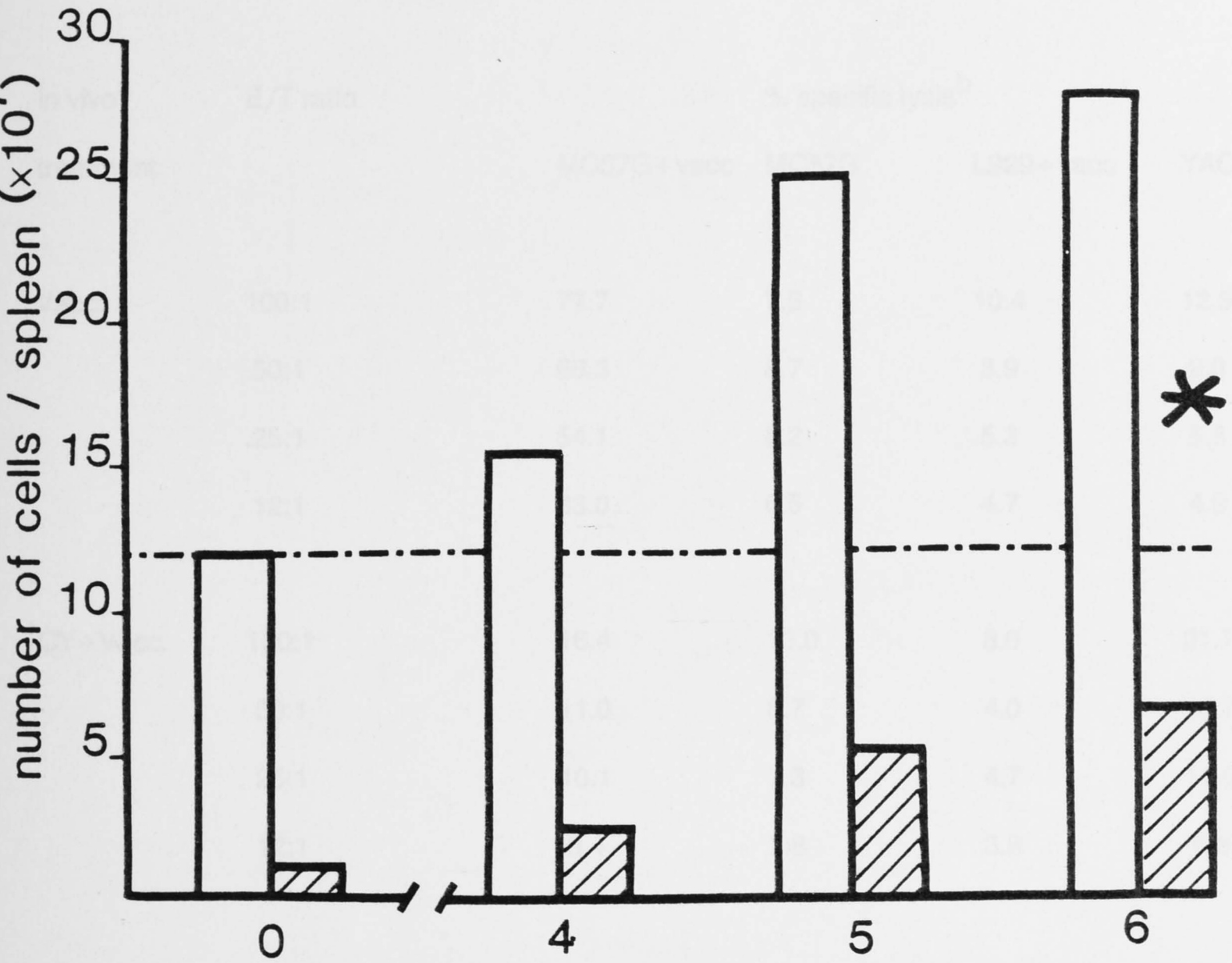


Fig. 3.3 Cellularity of spleens after virus infection

Mice were injected with virus only (☐) or following CY treatment (▨) as described at Fig. 3.2. Recovery of spleen cell number 8 days after immunosuppression in uninfected mice is represented by (*). Mean spleen cell number from 5-20 mice is shown.

Table 3.1 Primary cytotoxic activity of spleen cells after vaccinia infection

in vivo[a]	E/T ratio	% specific lysis[b]			
treatment		MC57G + vacc	MC57G	L929 + vacc	YAC
Vacc.	100:1	77.7	7.5	10.4	12.9
	.50:1	66.3	8.7	3.9	9.0
	.25:1	54.1	8.2	5.3	5.8
	12:1	33.0	6.5	4.7	4.9
CY + Vacc.	100:1	16.4	13.0	8.0	21.7
	50:1	11.0	8.7	4.0	16.7
	25:1	10.1	7.3	4.7	11.6
	12:1	8.4	7.8	3.8	8.6
Nil	100:1	8.3	8.9	4.7	9.0
	50:1	5.4	6.9	3.9	8.4
	25:1	5.8	5.	2.4	5.2
	12:1	5.2	9.0	2.8	4.0

a) B6 spleen cells tested 6 days after infection. In vivo treatment as described in Fig. 3.2.

b) 4 h ^{51}Cr-release assay for YAC targets, 6 h for the other targets.

Spontaneous release of targets: 31.2%, 18.4%, 23%, 21.6%.

Results are expressed as the mean of triplicate samples. S.E. of results per targets; < 5.5%, 3.0%, 3.6%, 3.5%.

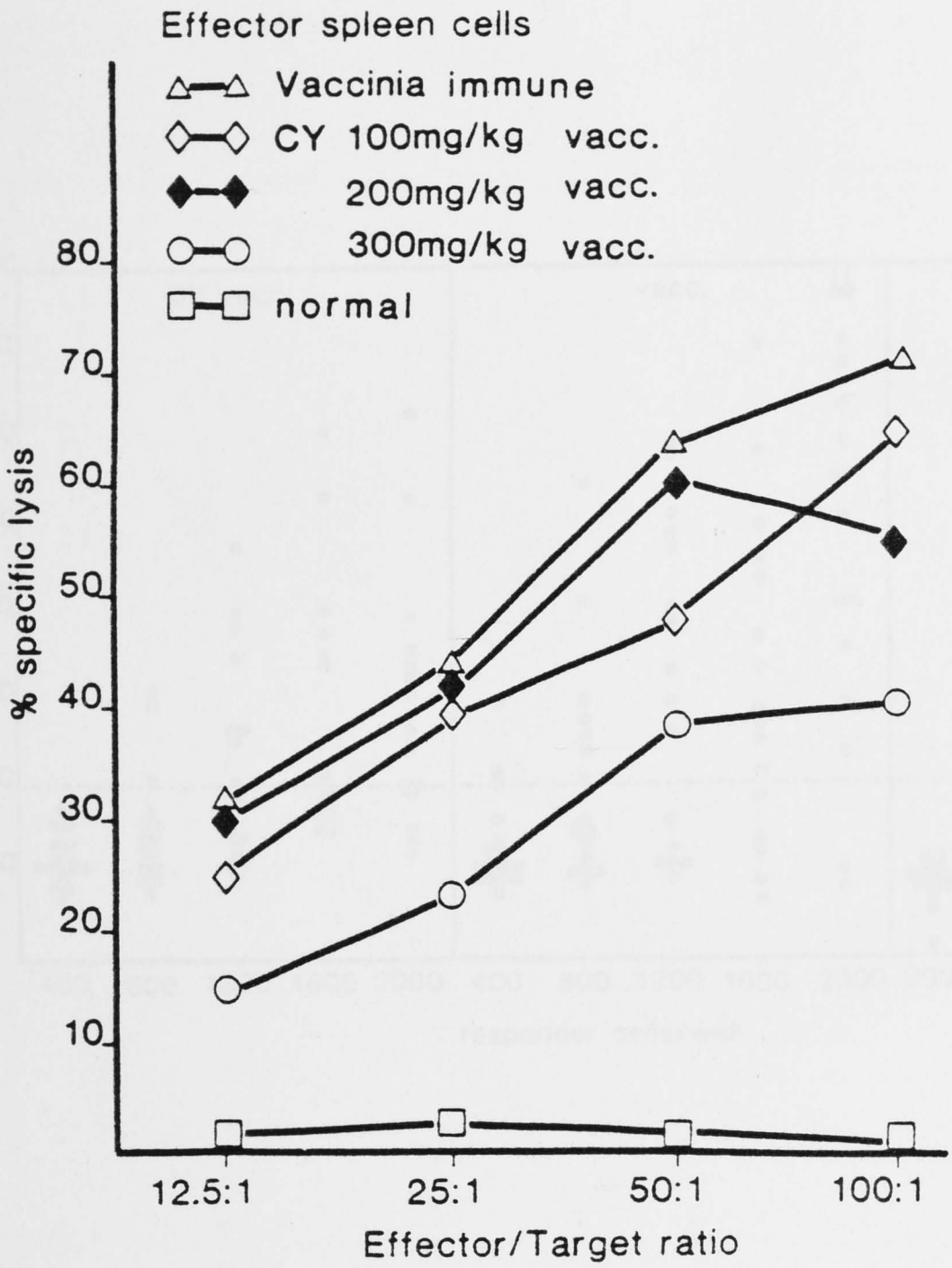


Fig. 3.4 Suppression of CTL activity by different amounts of CY.

B6 mice were treated in vivo as described at Fig. 3.2. Spleen cells were harvested 6 days after viral infection. Spontaneous release was 18.3% for vaccinia-infected MC57G targets and 19.6% for uninfected MC57G cells. The results are shown as mean of triplicate samples on infected targets, the S.E. being less than 5%. Cytotoxicity measured on uninfected targets was less then 10% in each group at 100:1 ratio, with S.E. being less than 1.5%.

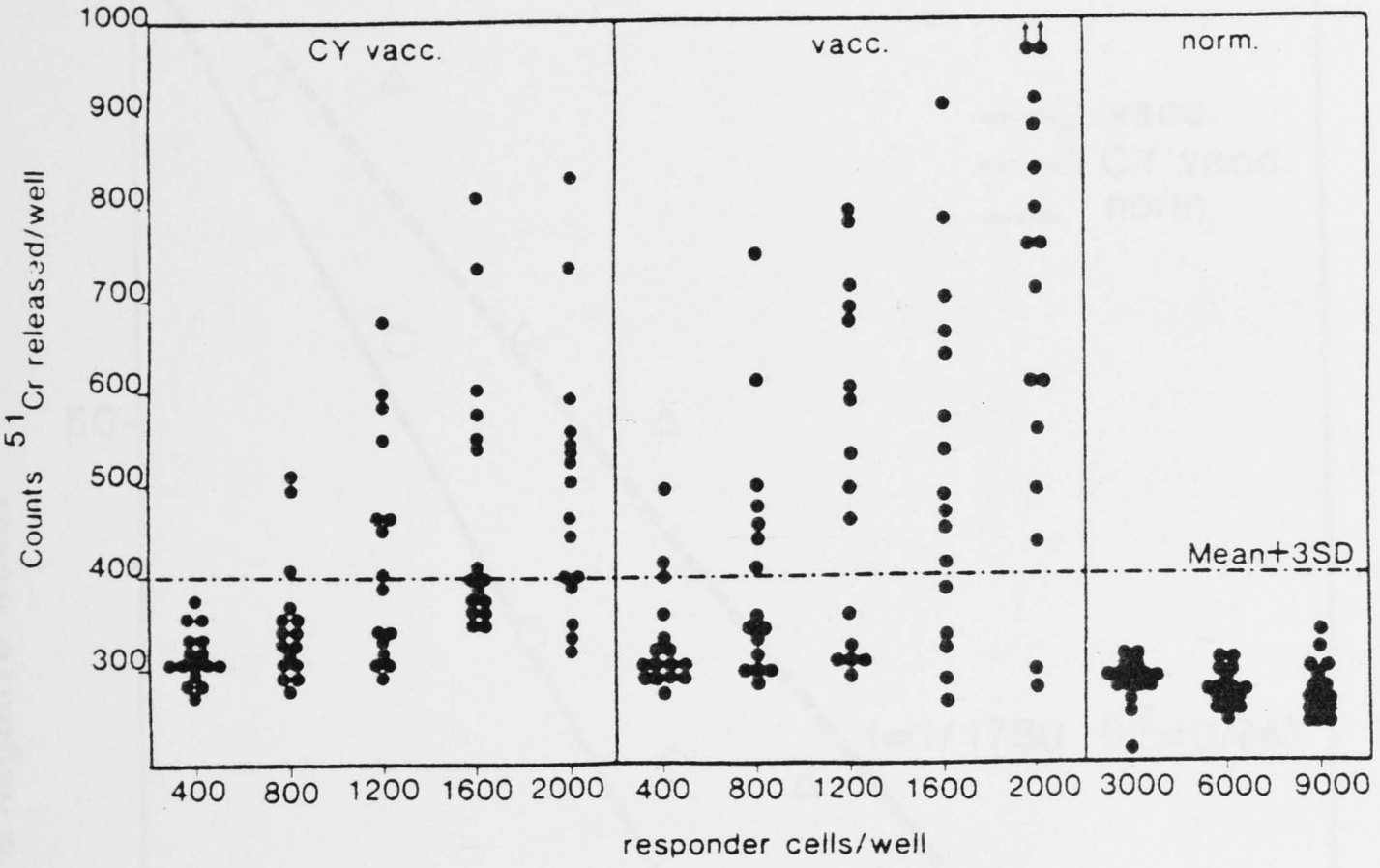


Fig. 3.5 Cytolytic activity of LDA microwells

Graded number of spleen cells from <u>in vivo</u> treated (as Fig. 3.2) or untreated animals were cultured with 10^5 T cell-depleted, virus-infected macrophages inactivated by UV irradiation, in 96 well trays for 8 days. Cytotoxicity from individual wells was measured on virus-infected MC57G target cells in an 8 hr ^{51}Cr-release assay.

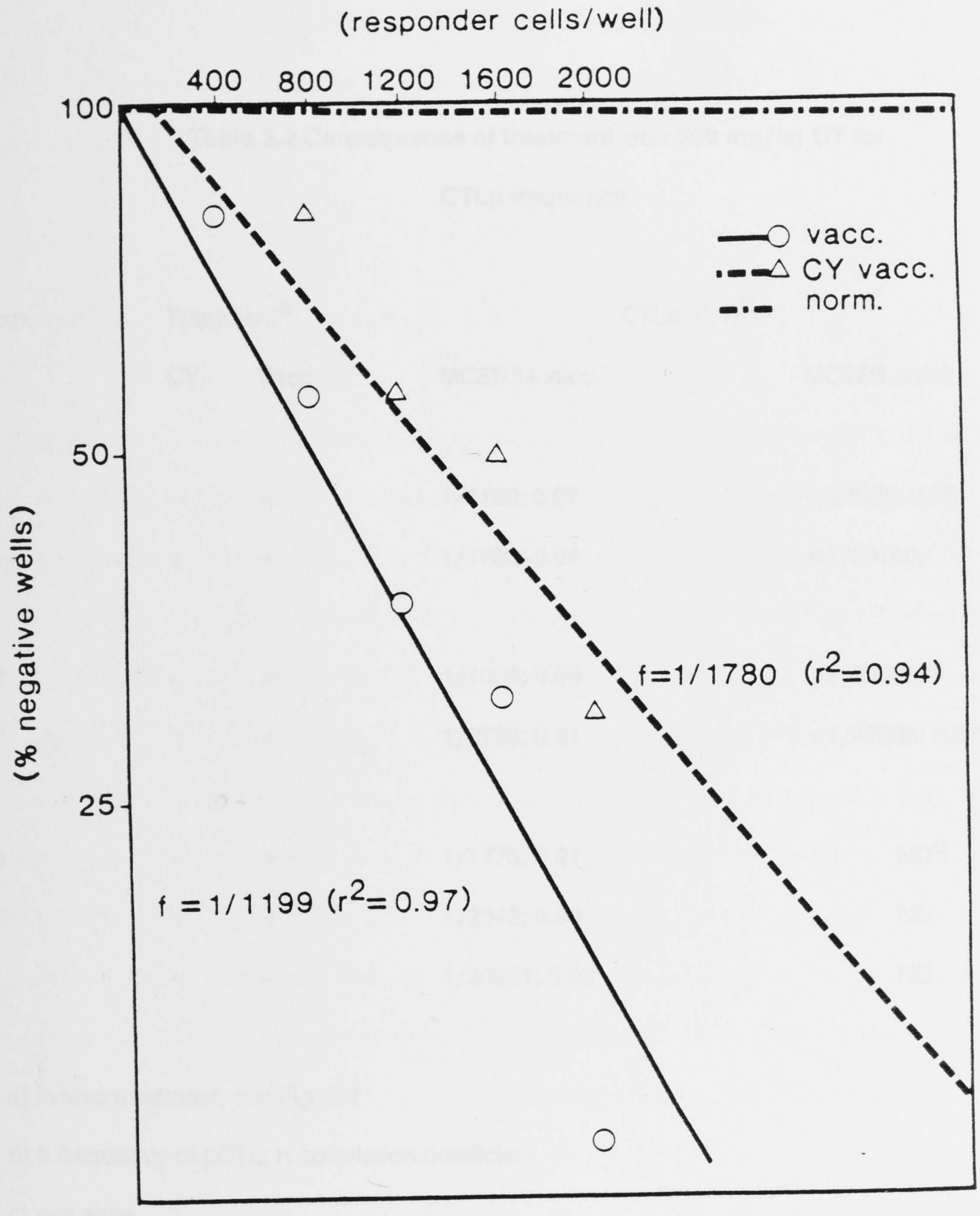


Fig. 3.6 Frequency of vaccinia-specific CTL-s

The frequency of pCTL was calculated by linear regression from the percentage of nonresponding wells expressed as log value (showing less than 3 x S.E. of cytotoxic activity from wells containing stimulator cells only, Fig. 3.5), (y axis) at each concentration of responder cells (16 wells/dilution) (x axis). Frequency of pCTL = 1, when 37% of the wells are negative for cytotoxicity by Poisson distribution.

Table 3.2 Consequence of treatment with 300 mg/kg CY for CTLp frequency.

Exp.	Treatment[a]		CTLp (f; r)[b]	
	CY	Vacc	MC57G + vacc.	MC57G uninf.
1	-	+	1/1199; 0.97	1/21938; 0.76
	+	+	1/1780; 0.94	<1/30700; -
2	-	+	1/1036; 0.86	<1/25000; -
	+	+	1/2733; 0.81	<1/47695; 0.87
3	-	+	1/1775; 0.91	ND[c]
	+	+	1/2343; 0.98	ND
	-	-	1/30921; 0.93	ND

a) In vivo treatment, see Fig. 3.2

b) f: frequency of pCTL; r: correlation coefficient

c) not done

becomes even more apparent (Table 3.3).Administration of CY diminishes the number of LU per spleen by a factor of 50-fold, while the decrease in CTLp per spleen is only 7-fold (Table 3.3). This difference reveals that the majority of CTLp fail to develop into effector T cells in vivo in the immunosuppressed animals.

3.2.4 Expression of cell surface glycoproteins

The results of the limit dilution assays suggest that no selective loss of the pCTL subset occurs due to the immuno-suppression. The ratio of different subsets of lymphocytes in the spleen of CY treated or untreated immune or normal mice was measured by flow microfluorometry (FMF). As a consequence of infection with vaccinia virus, the proportion of Thy-1$^+$ and Lyt-2$^+$ cells in the spleen is increased above the levels for normal controls, while there is little difference in the level of expression of L3T4$^+$ (top two panels, Fig. 3.8). However, the intensity of staining and the proportion of positive cells for all three cell-surface glycoproteins is greatly diminished on spleen cells from the CY-pretreated, vaccinia-infected mice (bottom panel, Fig. 3.8). This drop in the level of expression of Thy-1, Lyt 2 and L3T4 subsequent to CY-suppression and infection was confirmed in further experiments and was also found for mice that were given CY but no virus (Fig. 3.9). The decrease in Thy-1, Lyt 2 and L3T4 is not due to a relative increase in B cell numbers, as the second antibody used for Thy-1 staining (first column in Fig. 3.8) will also bind to mouse immunoglobulin: any B cells would thus be included in the background staining for Thy-1 (Fig. 3.8).

The fact that individual cells are expressing less Thy-1, Lyt 2 and L3T4 in the drug-treated mice was further demonstrated by culturing spleen cells in vitro under conditions that cause selective proliferation (Ceredig, 1986; Erard, 1985) of all T lymphocytes. Again, after 4 days in culture, FMF profiles for lymphocytes from mice that were given CY alone, or CY + virus, show much lower levels of Lyt 2 and L3T4 (Fig. 3.10), though the effect on Thy-1 is not quite so apparent as that found for cells taken directly from spleen (compare Fig. 3.10 with 3.8 & 3.9). The overall conclusion is, however, that CY-treatment causes decreased expression of Lyt 2, L3T4 and Thy-1, with this effect

Table 3.3 Effect of CY-immunosuppression on vaccinia-immune cytotoxic activity and pCTL frequency

300 mg/kg Cy[a]	Lytic units[b] per 10^7 cells	spleen	vaccinia-targets		CTLp[c] frequency	
						uninfected
			f	r^2	No./spleen	f
-	395	11266	1/1199	0.97	23.8×10^4	>1/21938
+	39	221	1/1780	0.94	3.2×10^4	>1/30700

a) Cy was given 2 days before virus. Cells were harvested 6 days after infection.

b) Calculated as the number of cells necessary to cause 30% specific lysis of vaccinia virus-infected MC57G targets in a 6hr ^{51}Cr release assay.

c) Determined by culturing the 6 d immune spleen cells under limiting dilution conditions for a further 8d in the presence of excess IL-2.

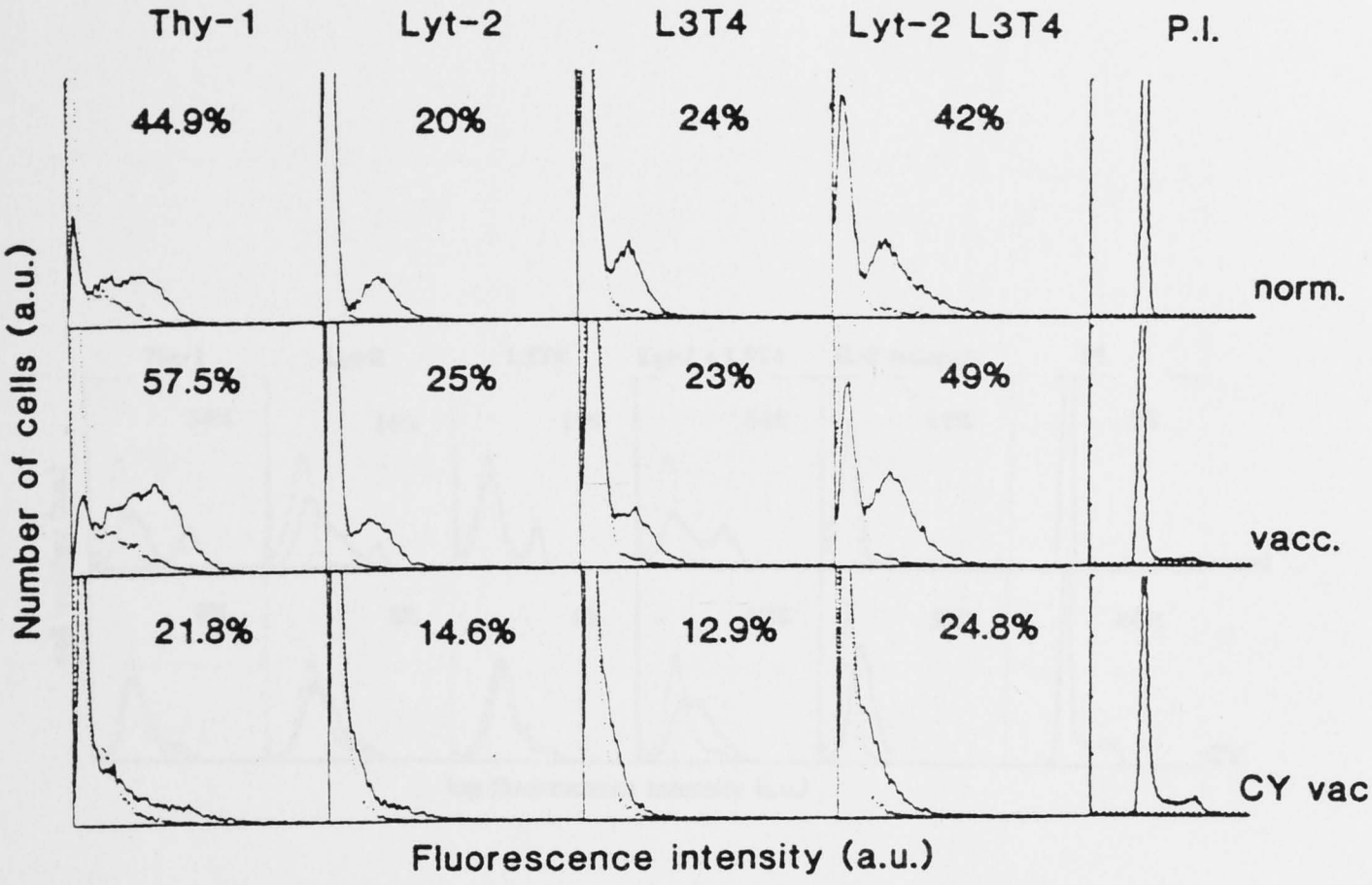


Fig 3.8 Phenotypic analysis of freshly isolated spleen cells

Animals were treated as described in Fig 3.2 and the phenotype of spleen cells was analysed 6 days after infection. The mAb to Lyt 2 (53.6.7) and L3T4 (H-129) but not Thy-1 (AT83) were biotinylated. The dotted lines represent the binding of second antibody (sheep anti-rat FITC for Thy-1 and avidin-FITC for the others). The percent value represents the difference between the control (staining with second antibody) and experimental results. The last column shows the ratio of cycling cells stained with propidium iodide (P.I.). a.u. = arbitrary units

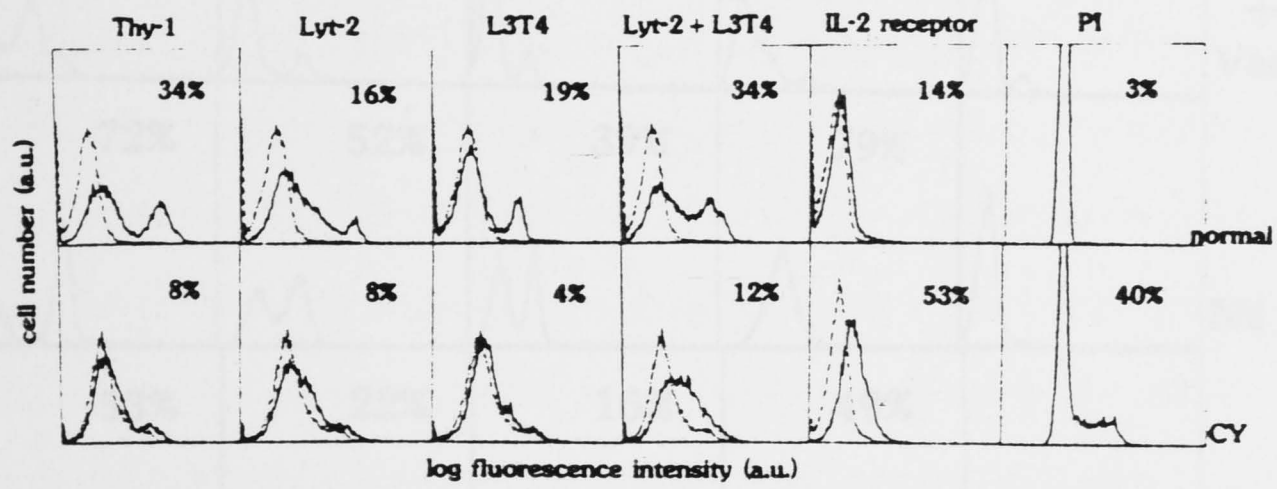


Fig. 3.9 Phenotypic analysis of freshly isolated spleen cells.

Spleen cells were tested from normal animals (first row) and from immunosuppressed mice treated with 300 mg/kg CY 8 days before analysis. Biotinylated antibodies (AT83 for Thy-1, 53.6.7 for Lyt 2, H-129 for L3T4 and PC61 for IL-2 receptor) followed by labelling with avidin-FITC were used. The percent of stained cells is expressed after subtracting the value of control samples stained with second antibody alone (dotted line). DNA content was determined by P.I.staining.

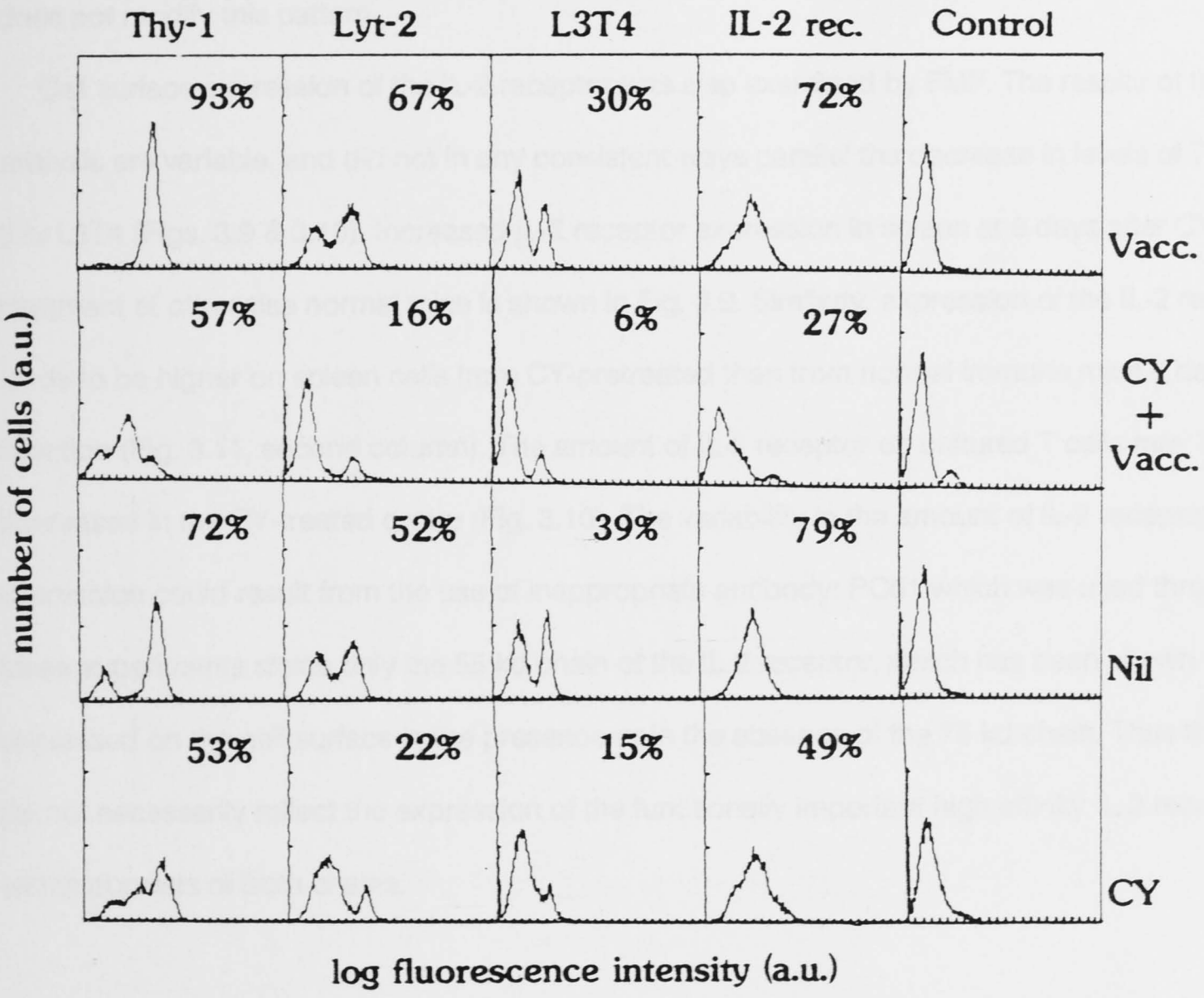


Fig. 3.10 Phenotypic analysis of spleen cells after mitogenic stimulation

Mice were treated as described at Fig. 3.2. Spleen cells were incubated for 4 days in the presence of 100 ng/ml Cal, 1ng/ml PMA and 10 U/ml EL-4 supernatant. Staining for FMF analysis was performed as described previously (Figs. 3.8 & 3.9). Control samples stained with the second antibody are shown in the last column.

persisting through successive cycles of cell division. The additional involvement of vaccinia virus does not modify this pattern.

Cell surface expression of the IL-2 receptor was also examined by FMF. The results of this analysis are variable, and did not in any consistent ways parallel the decrease in levels of Thy-1, Lyt 2 or L3T4 (Figs. 3.9 & 3.10). Increased IL-2 receptor expression in spleen at 8 days after CY-treatment of otherwise normal mice is shown in Fig. 3.9. Similarly, expression of the IL-2 receptor tends to be higher on spleen cells from CY-pretreated than from normal immune mice 6 days after infection (Fig. 3.11, second column). The amount of IL-2 receptor on cultured T cells may be decreased in the CY-treated group (Fig. 3.10). The variability in the amount of IL-2 receptor expression could result from the use of inappropriate antibody: PC61 which was used throughout these experiments stains only the 55 kd chain of the IL-2 receptor, which has been shown to be expressed on the cell surface in the presence or in the absence of the 75 kd chain. Thus the results do not necessarily reflect the expression of the functionally important high affinity IL-2 receptor, which consists of both chains.

3.2.5 Restoration of CTL activity

A major difference between the assays for CTL function and CTLp frequency is that the CTLp are cultured in vitro in the presence of excess IL-2. There is ample evidence from other experimental systems that the immunodeficiency associated with CY treatment may, at least in part, be corrected by administering IL-2 either in vitro or in vivo (Ballas, 1986; Merluzzi et al. 1983 & 1984; Walker et al. 1985). Injection of mice with a single dose of IL-2 causes an obvious increase in primary cytotoxicity (Table 3.4 & Fig. 3.12) but augments pCTL frequency by a factor of less than two-fold (Table 3.4).Similarly, bulk culture of spleen cells in vitro in the presence of added IL-2 results in enhanced CTL activity (Fig. 3.13) with a very small increase in NK-like or LAK activity tested on uninfected syngeneic or YAC targets (Fig. 3.14).

Stimulation of the virus-immune cells with PMA & CaI in the presence of IL-2 (the protocol is known to stimulate cytolytic activity of primed T cells while maintaining their original specificity,

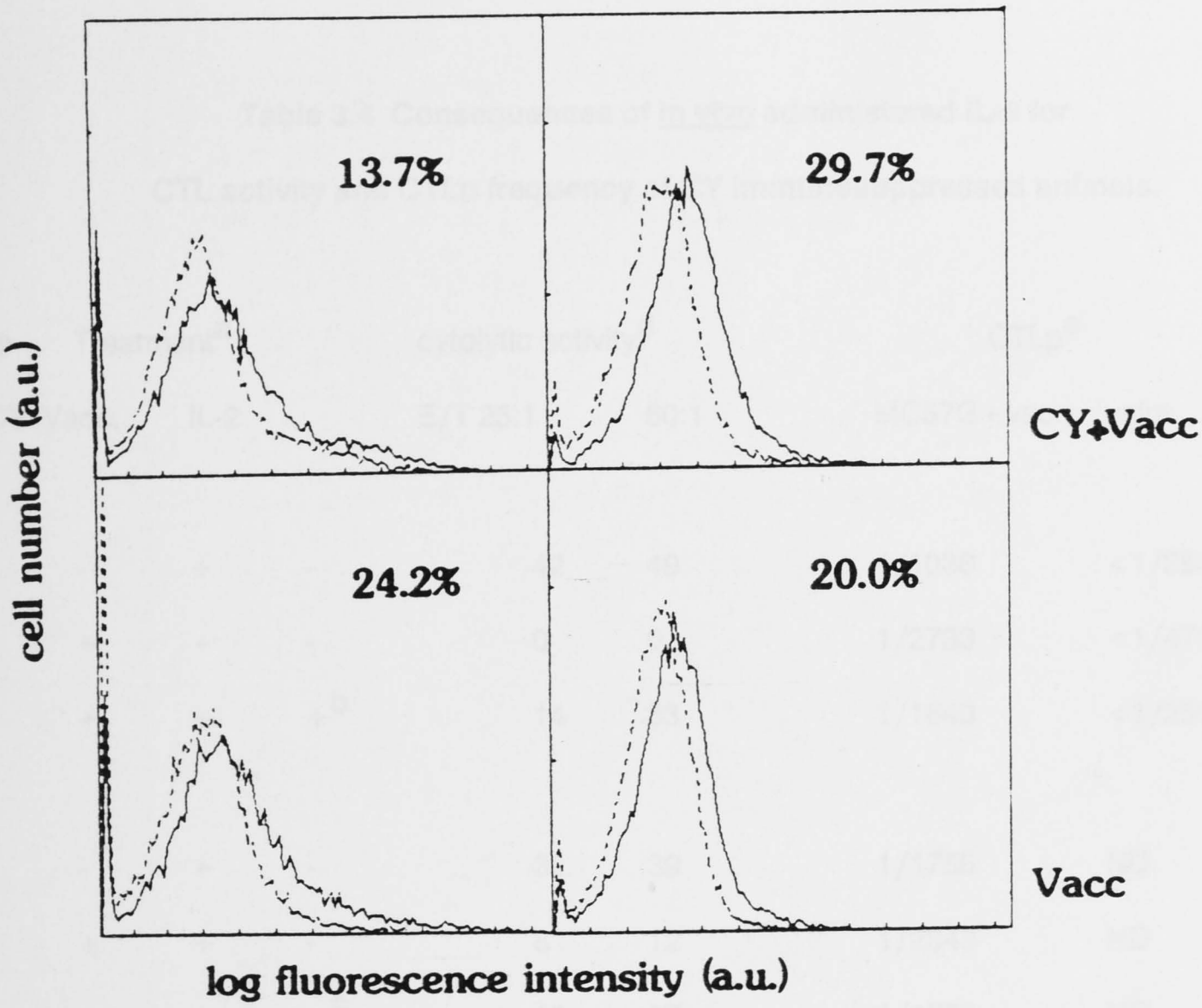


Fig. 3.11 IL-2 receptor expression on freshly isolated spleen cells

Mice were treated with CY and vaccinia (first row) or with the virus only (second row) as described (Fig. 3.2). Spleen cells were harvested 3 days (first column) or 6 days (second column) after infection with virus. Cells were stained with biotinylated PC61 antibody and avidin-FITC. The percent stained cells was calculated as in Figs. 3.8 and 3.9.

Table 3.4 Consequences of <u>in vivo</u> administered IL-2 for CTL activity and CTLp frequency of CY immunosuppressed animals.

Exp	Treatment[a]			cytolytic activity[d]		CTLp[e]	
	CY	Vacc.	IL-2	E/T 25:1	50:1	MC57G+vacc	uninf.
1	-	+	-	42	49	1/1036	<1/25000
	+	+	-	0	0	1/2733	<1/47000
	+	+	+[b]	14	33	1/1640	<1/25000
2	-	+	-	32	39	1/1755	ND
	+	+	-	8	12	1/2343	ND
	+	+	+[c]	13	17	1/1780	ND

a) <u>in vivo</u> treatment see Fig. 3.2. IL-2 was injected i.p. 4 hrs before infection with virus.

b) 800 U/mouse, EL-4 sn

c) 200 U/mouse, human recombinant IL-2

d) Effector cells were harvested 6 days after virus infection and their lytic activity was measured on vaccinia- infected MC57G target cells in a 6 hr ^{51}Cr-release. Spontaneous release is 22%, S.E. < 5%.

e) calculated by linear regression as described in Fig. 3.6.

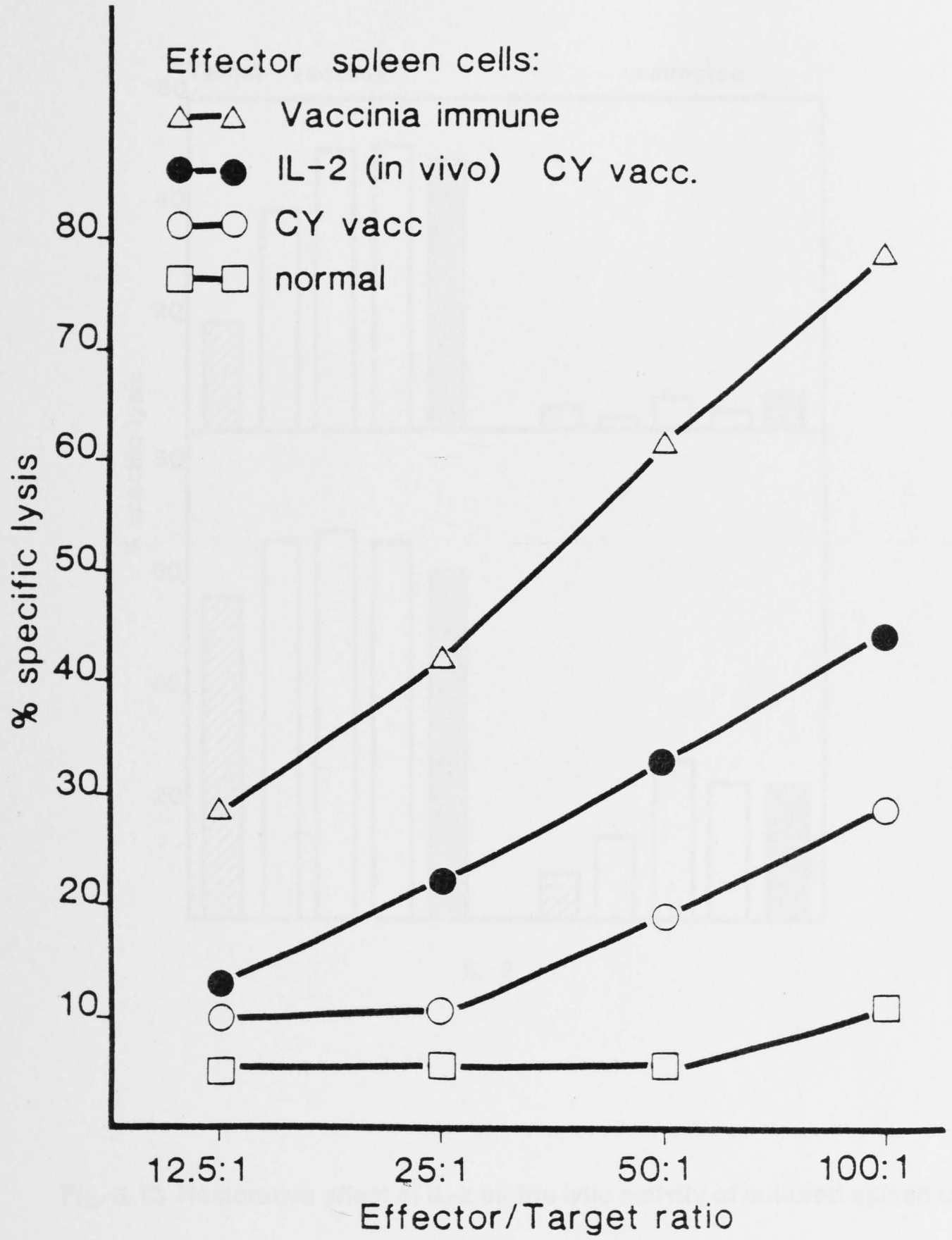


Fig. 3.12 Restorative effect of <u>in vivo</u> administered interleukin-2.

Mice were treated <u>in vivo</u> as described at Fig. 3.2 and one group (●——●) was also injected with human recombinant IL-2 i.p., 4 hours before the virus. Cytotoxic activity of spleen cells 6 days after priming on virus-infected MC57G targets is shown. Spontaneous release = 31%, S.E. < 5%.

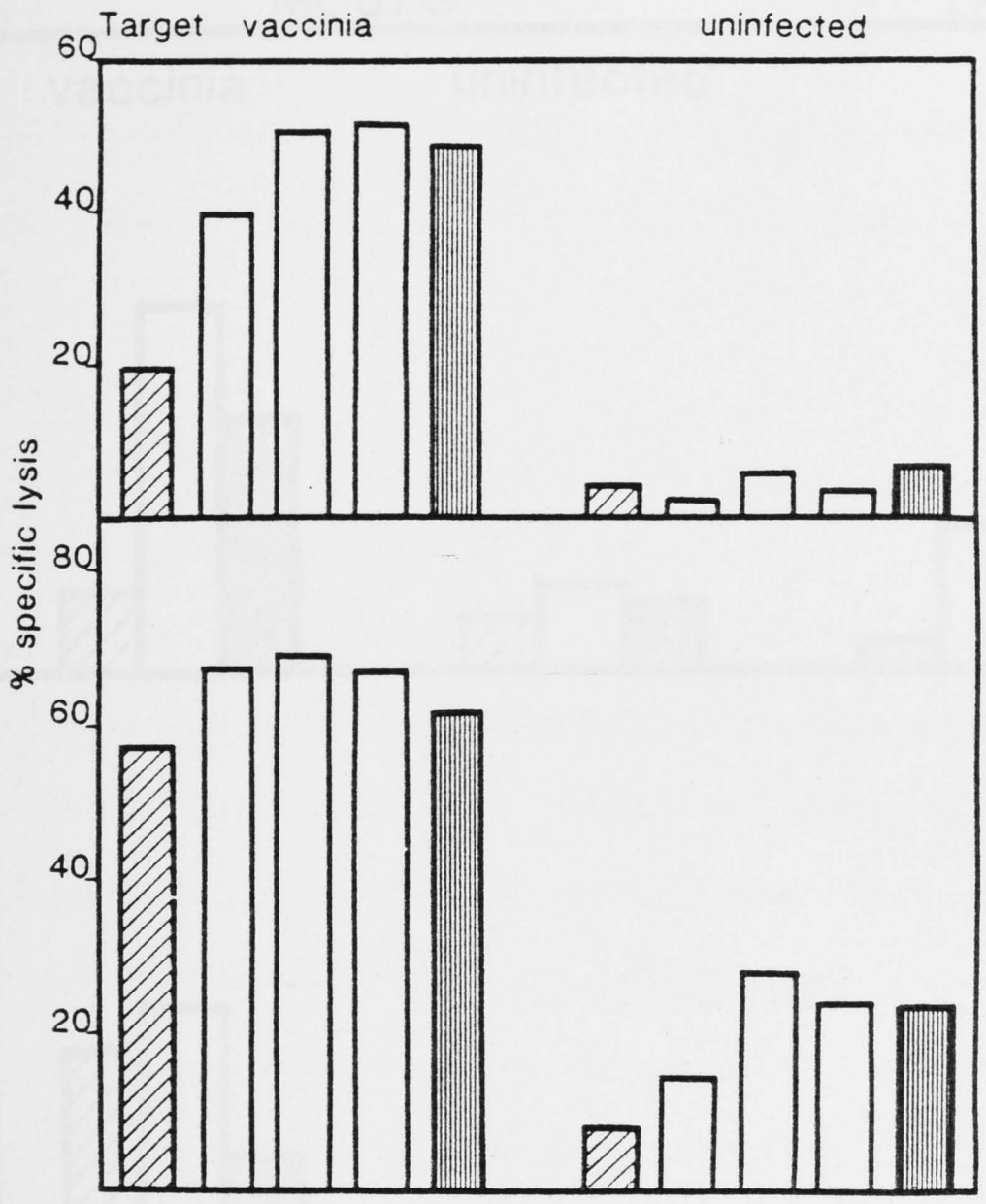


Fig. 3.13 Restorative effect of IL-2 on the lytic activity of cultured spleen cells.

Vaccinia-immune spleens from normal (B) or CY-treated (A) mice were incubated with virus- infected stimulator cells as described at Fig. 3.5, in the absence (▨) or in the presence (□) of graded amounts of EL-4 sn (20, 40, 60 U/ml) or 25 U/ml highly purified human recombinant IL-2 (▥)for 4 days. Vaccinia-infected (1-5 col.) or uninfected (6-10 col.) MC57G cells were used as targets at 40:1 effecto: target ratio. The results are expressed as the mean of triplicate samples, the S.E. being less than 5%.

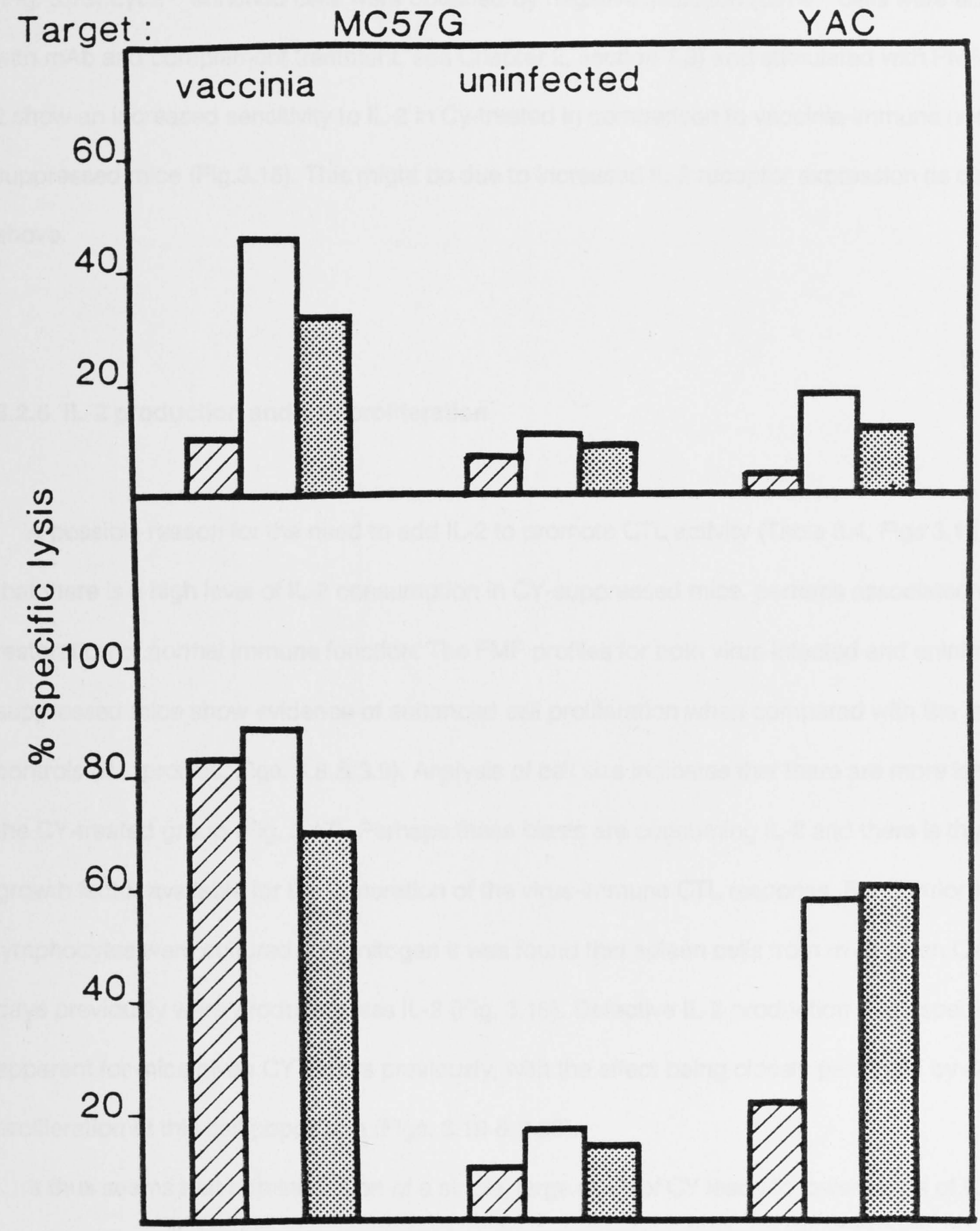


Fig. 3.14 Restorative effect of IL-2 on the lytic activity of cultured spleen cells

Normal (A) and CY-treated (B) immune spleen cells were cultured and tested for cytotoxicity as described in Fig. 3.13. Non-specific activity was also tested on NK-sensitive YAC target cells (last column). Cells were grown in vitro in the absence (▨) or in the presence of 50 U/ml (□) or 100 U/ml (▩) IL-2. Effector:target ratio was 50:1.

Chapter 4) also greatly increases (by a factor of 9.1) the CTL activity of CY pretreated spleen cells (Fig. 3.15).Lyt 2^+ enriched cells were obtained by negative selection ($L3T4^+$ cells were eliminated with mAb and complement treatment, see Chapter 2, section 7.3) and stimulated with PMA+Cal+IL-2 show an increased sensitivity to IL-2 in Cy-treated in comparison to vaccinia-immune non-suppressed mice (Fig.3.16). This might be due to increased IL-2 receptor expression as discussed above.

3.2.6 IL-2 production and cell proliferation

A possible reason for the need to add IL-2 to promote CTL activity (Table 3.4, Figs 3.13 - 3.16) is that there is a high level of IL-2 consumption in CY-suppressed mice, perhaps associated with the restoration of normal immune function. The FMF profiles for both virus-infected and uninfected CY-suppressed mice show evidence of enhanced cell proliferation when compared with the relevant controls (P.I. profiles, Figs. 3.8 & 3.9). Analysis of cell size indicates that there are more large cells in the CY-treated group (Fig. 3.17). Perhaps these blasts are consuming IL-2 and there is thus less growth factor available for the generation of the virus-immune CTL response. Furthermore, when lymphocytes were cultured with mitogen it was found that spleen cells from mice given CY 4 or 8 days previously were producing less IL-2 (Fig. 3.18). Defective IL-2 production was especially apparent for mice given CY 4 days previously, with the effect being closely paralleled by decreased proliferation of this cell population (Figs. 3.19 & 3.20).

It thus seems that administration of a single, large dose of CY leads to lower levels of IL-2 production and diminished cell-surface expression of Thy-1, Lyt 2 and L3T4. These effects persist for at least 8 days of drug treatment and interfere with the ability of lymphoid cells to develop a CTL response.

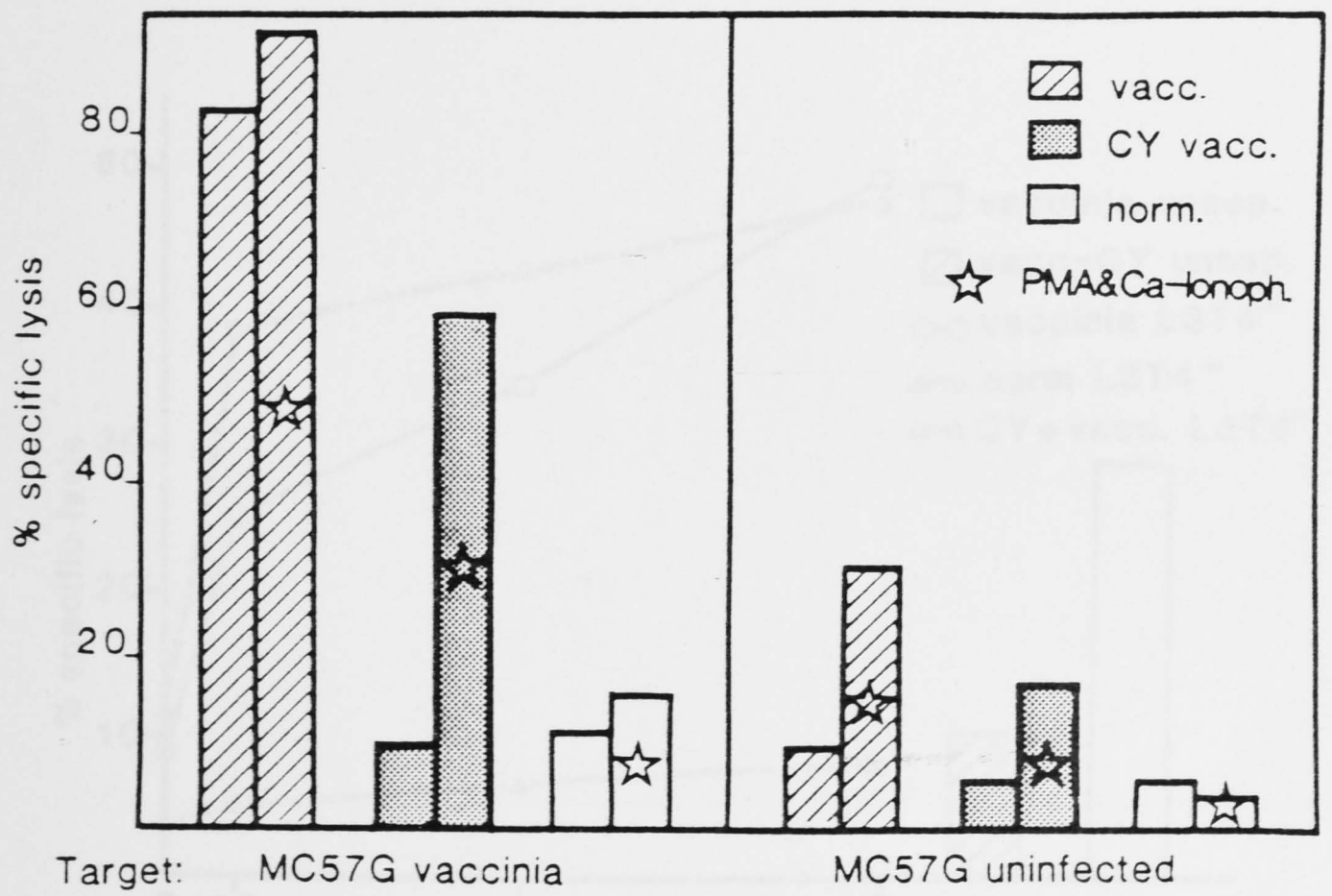


Fig. 3.15 Restorative effect of PMA & CA-I on lytic activity.

Spleen cells from normal mice ( ); mice treated with vaccinia (or with CY and vaccinia ( ) (as described in Fig. 3.2) were harvested 6 days after infection and cultured for 4 days in the presence or absence of 3 ng/ml PMA, 500 ng/ml CaI and 10 U/ml IL-2. Cytotoxic assay was performed as described. Spontaneous release for targets: 25.2% and 24.0%, respectively. E/T ratio = 40:1, S.E. < 5%.

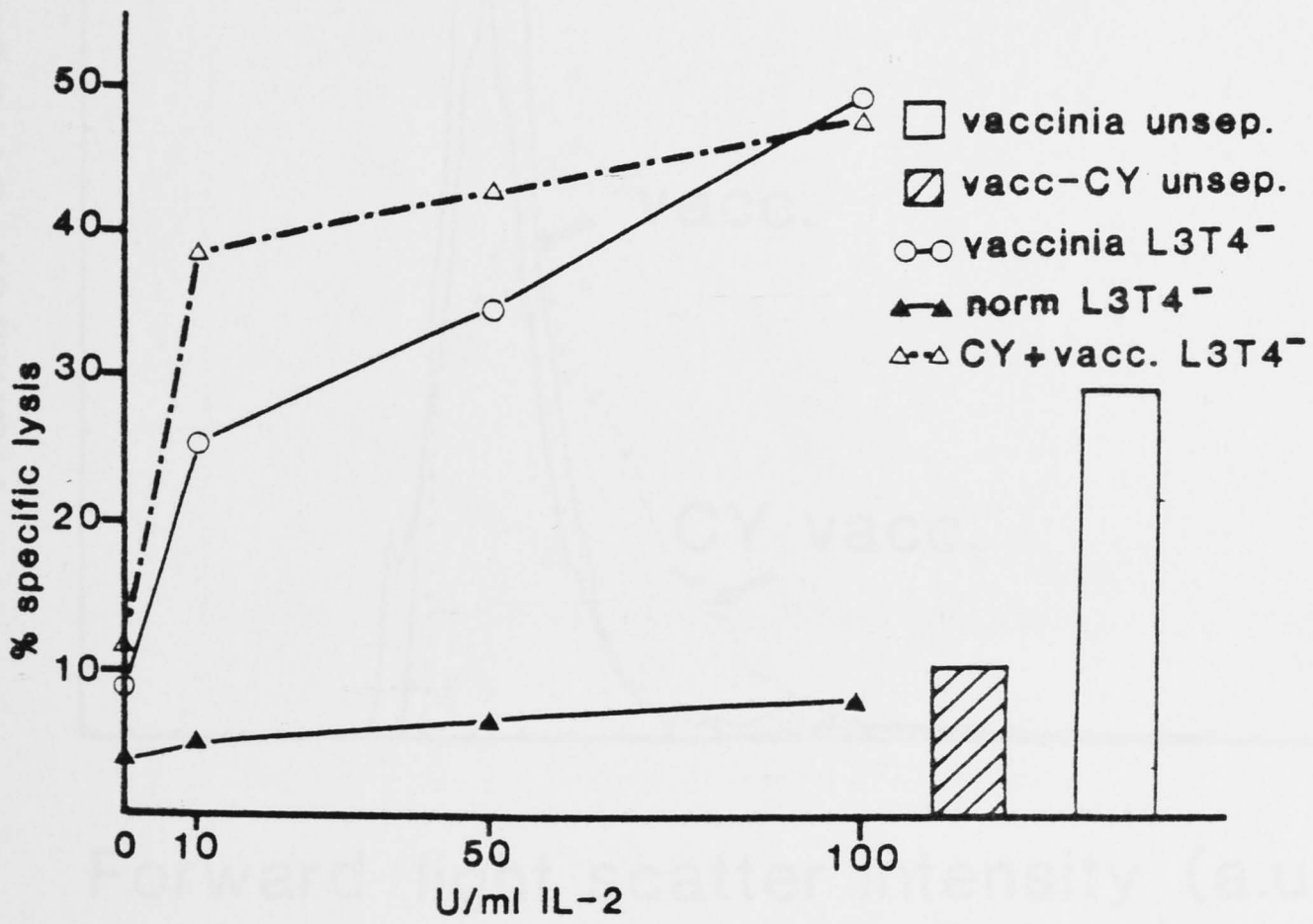


Fig. 3.16 Restoration of the lytic function of the Lyt 2^+ subset by IL-2.

Spleen cells from immunosuppressed or non-suppressed virus-immune mice were treated with RL 172 (anti-L3T4) antibody and complement. The effectiveness of depletion was checked by FMF (contamination with $L3T4^+$ cells was < 1.0%). Cells were then incubated in the presence of 1 ng/ml PMA, 100 ng/ml Cal and in the presence or absence of graded amounts of EL-4 sn for 3 days. Cytotoxicity on vaccinia-infected MC57G targets was measured in ^{51}Cr-release assay at different E:T ratios. The % specific lysis at 15:1 E:T ratio is shown, calculated by linear regression of the slopes (S.E. < 4.7%, r >0.9). Killing of uninfected MC57G targets was always less than 5%.

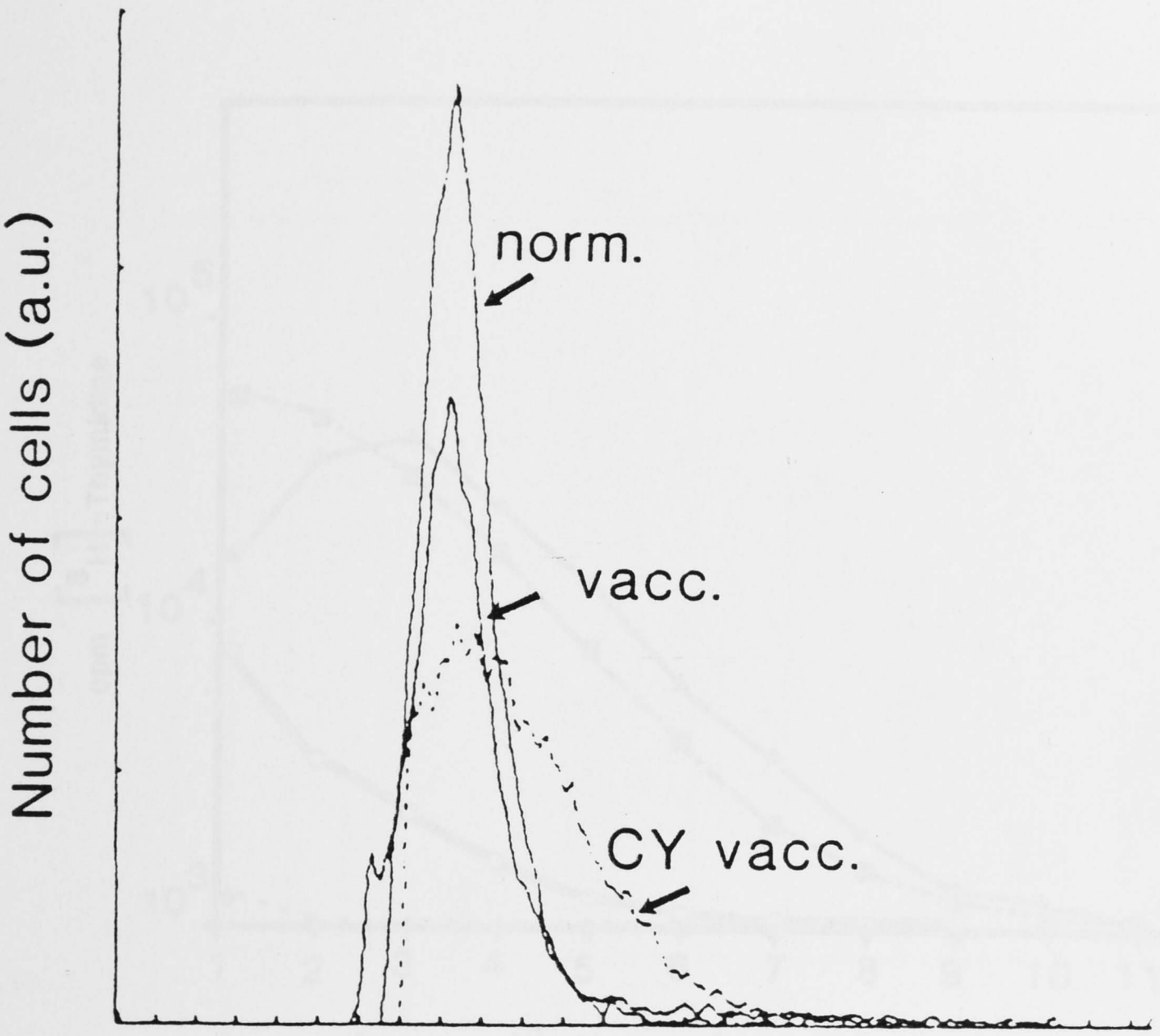


Fig. 3.17 Relative cell size after CY treatment

Spleen cells from normal or in vivo treated (Fig. 3.2) mice were analysed by FMF. Forward light scatter intensity (FLS) is shown on the figure versus cell number, reflecting the presence of more blasts cells in the two CY-treated groups.

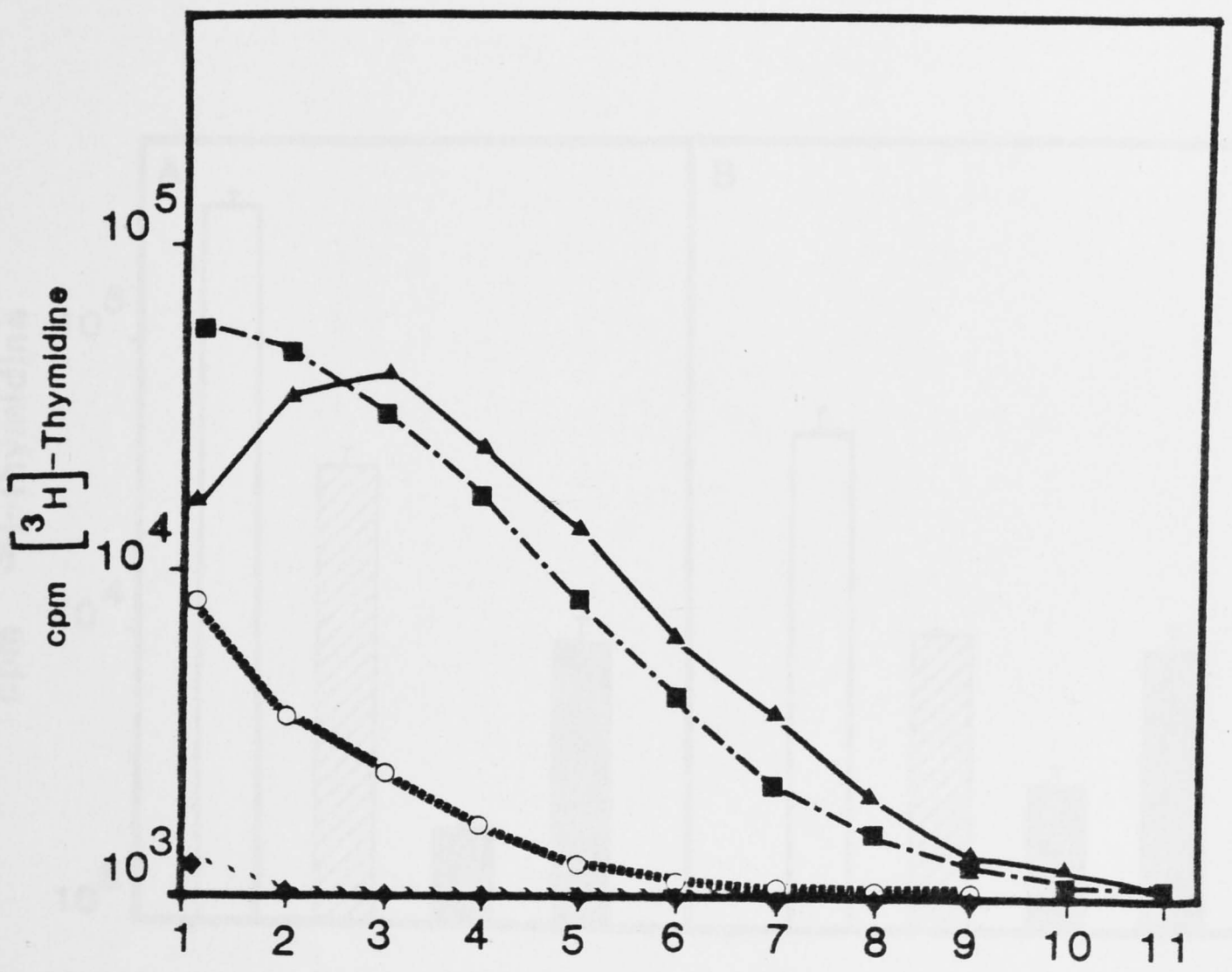


Fig. 3.18 Effect of CY treatment on IL-2 production upon mitogenic stimulation

Mice were treated with 300 mg/kg CY and their spleen removed 2 (▲——▲), 4 (◆···◆) or 8 days (○----○) after treatment. Untreated control (■—·—■). Cells were incubated with 1 ng/ml PMA and 100 ng/ml CaI for 2 days at 10^5 cells/well then 50 μl supernatant was removed from each well, serially diluted and tested for the capacity to induce proliferation of IL-2 dependent CTLL cells in a 24 h assay.

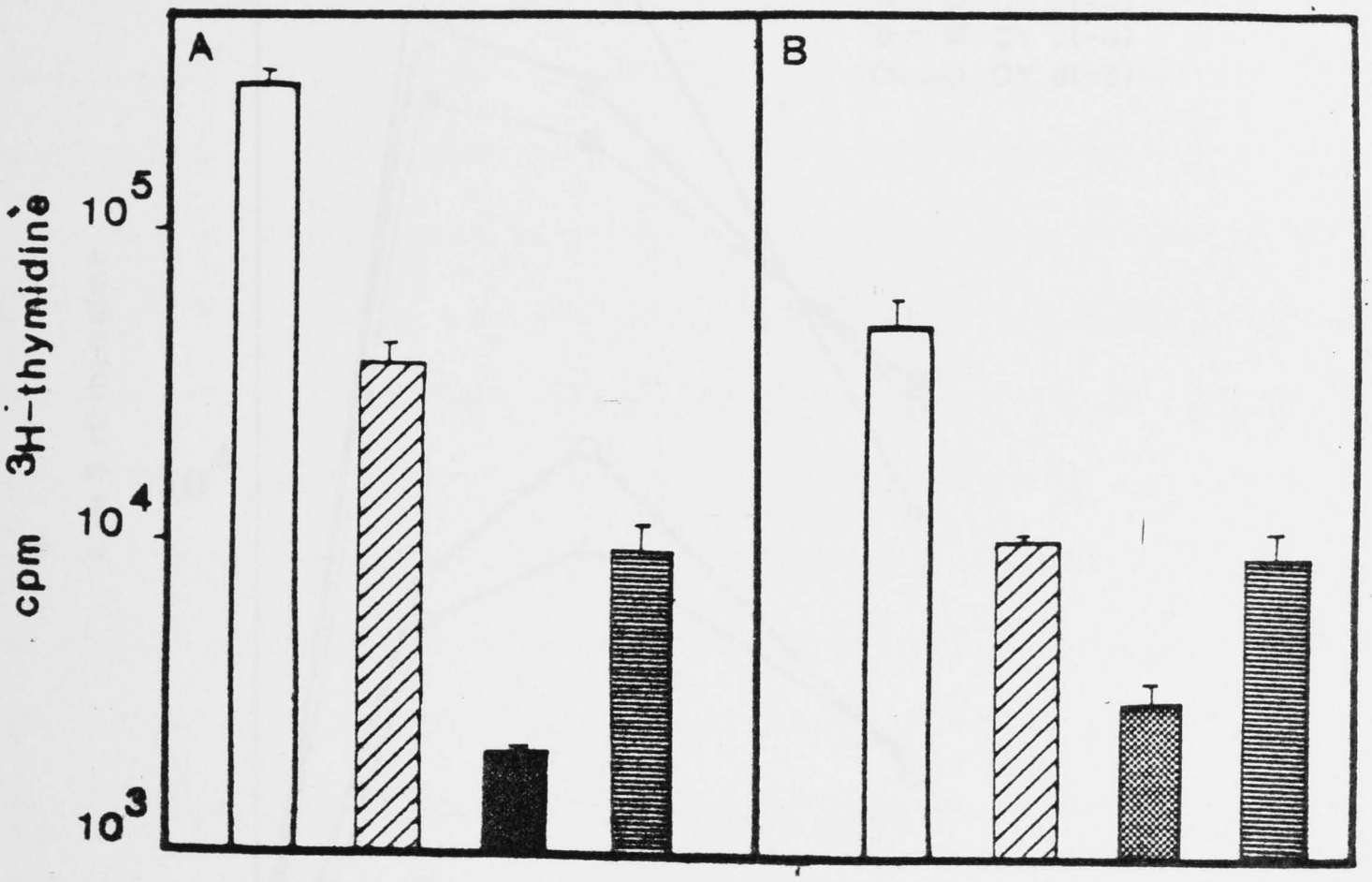


Fig. 3.19 Effect of CY-treatment on mitogen induced proliferation

Spleen cells from normal ( ) or CY treated mice (injected 2 days , 4 days or 8 days before the assay) were treated with 1 ng/ml PMA and 100 ng/ml CaI (A panel) or with 3 μg/ml Con A (B panel) for 2 days. No exogeneous IL-2 was provided. Cells were pulsed with 1 μCi/well [^{3}H]-thymidine for 6 hrs and thymidine uptake was measured as described in Chapter 2, section 11.1.

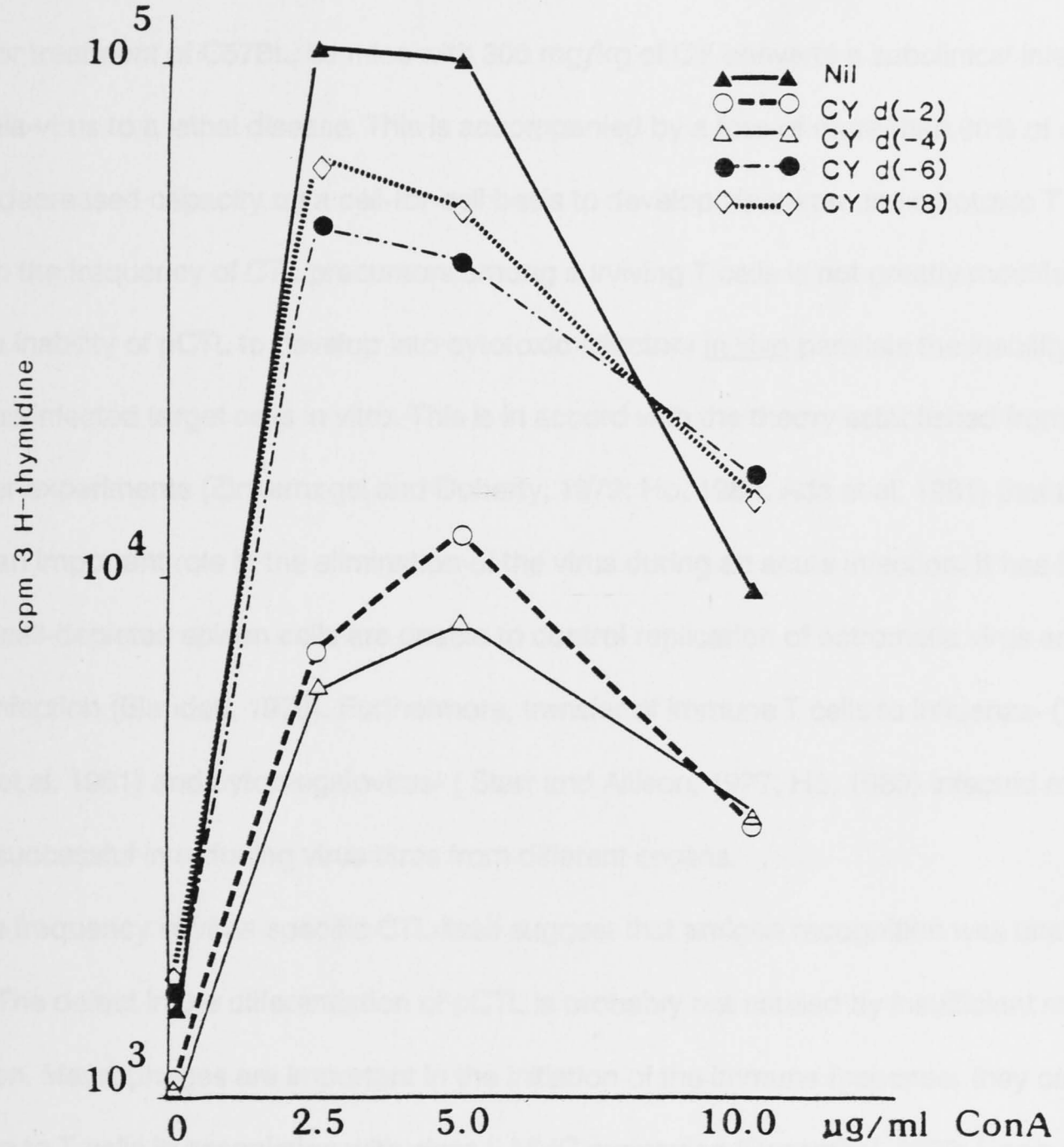


Fig. 3.20 Con A stimulation of CY treated spleen cells

Untreated (▲—▲) or CY-treated spleen cells (injected 2 days, O— —O ; 4 days, △—△ ; 6 days ●—·—·● or 8 days ◇''''''◇ before the assay) were stimulated with increasing amount of Con A for 72 hours. Cells were pulsed with [^{3}H]-thymidine for the last 6 hrs of the incubation and the isotope uptake was measured as described. Mean of triplicate samples is shown.

3.3 Discussion

Prior treatment of C57BL/6J mice with 300 mg/kg of CY converts a subclinical infection with vaccinia-virus to a lethal disease. This is accompanied by a loss of more than 80% of spleen cells and a decreased capacity on a cell-for-cell basis to develop virus-immune cytotoxic T lymphocytes, though the frequency of CTL precursors among surviving T cells is not greatly modified.

The inability of pCTL to develop into cytotoxic effectors in vivo parallels the inability of T cells to kill virus-infected target cells in vitro. This is in accord with the theory established from adoptive transfer experiments (Zinkernagel and Doherty, 1979; Ho, 1980; Ada et al. 1981) that the CTL subset plays an important role in the elimination of the virus during an acute infection. It has been shown that T cell-depleted spleen cells are unable to control replication of ectromelia virus and recovery from infection (Blanden, 1970). Furthermore, transfer of immune T cells to influenza- (Yap et al. 1978; Wells et al. 1981) and cytomegalovirus- (Starr and Allison, 1977; Ho, 1980) infected mice has proven to be successful in reducing virus titres from different organs.

The frequency of virus-specific CTL itself suggest that antigen recognition was unaffected by the drug. The defect in the differentiation of pCTL is probably not caused by insufficient macrophage function. Macrophages are important in the initiation of the immune response: they can present antigen to T cells in association with class II MHC molecules (Rosenthal, 1978; Unanue, 1981) and release IL-1, a co-factor, necessary for T cell stimulation (Oppenheim et al. 1979; Farrar et al. 1980; Smith et al. 1980b; rev. by Durum et al. 1985). In addition it has been shown that they are most susceptible to CY-treatment immediately after administration of the drug (Schlick et al. 1985) and are able to contribute substantially to the development of a virus-induced inflammatory process whithin 3 or 4 days of drug treatment (Ceredig et al. 1987). Thus, the early steps in the development of T cell response seem to be less affected in our model.

The most obvious explanation for the inhibited differentiation of pCTL is the lack of T cell growth factor, interleukin 2, which has been shown to be essential and sufficient to mediate differentiation of T cell precursors (Erard et al. 1985a and b; Smith, 1980a; also see Chapter 1, Section 4.3). Experiments in vitro with "deactivated" T cell clones have shown that provision of IL-2 induces cytolytic capacity (Orosz et al. 1985). Also, in vivo reconstitution of immunocompromised mice with

IL-2 restored their immune response against pichinde (Walker et al. 1985) and influenza A (Merluzzi et al. 1984) viruses. Our results, that spleen cells from CY-suppressed, uninfected mice produce substantially less IL-2, especially at the stage when T cell differentiation should occur (>3days after infection), provides an explanation for the relative lack of cytotoxic activity following CY-suppression and infection with vaccinia virus. However the recovery is only partial, even when high amounts of IL-2 are provided in vitro or large doses of IL-2 were given 3 times in vivo (data not shown).

As the L3T4$^+$ population is the major subpopulation of cells which produces interleukin 2 and also other lymphokines, it seems likely that production of interferon γ, IL-4 and other factors is also affected. Interferons play a very important role in anti-viral defence, as will be discussed in Chapter 7, (Stewart, 1981; Nathan et al. 1983) and endogeneously produced IFN γ is probably also important in T-cell differentiation (Klein and Bevan, 1982; Simon et al. 1986).

The spleen cells from Cy-treated mice express low levels of cell surface Thy-1, Lyt 2 and L3T4 and this defect is inherited through successive generations, as lymphocytes proliferate either during the response to virus or as a consequence of exposure to mitogens (Allan and Doherty, 1985; Denizot et al. 1986). This may reflect defective, or diminished, synthesis of these glycoproteins. As discussed in Chapter 1 (Section 1.2), the Lyt-2 and L3T4 molecules play an important role in T cell - antigen recognition, as enhancers of specific cell-cell interaction and also as components of multi-molecular complexes mediating transduction of signals. Thus, decreased amounts of these molecules, expressed on the surface of T cells could lead to insufficient binding to the APC or target cells, resulting in impaired effector function.

The exact mechanism governing low T cell surface glycoprotein expression following CY treatment is not known, though this could result from the capacity of CY derivatives to cause genetic lesions (Takeshita and Conner, 1984; Wilmer et al. 1984). Cells may survive damage to regions of DNA which are not essential for the basic replicative mechanism of the cell. Downregulation of T cell antigens has also been shown, following non-cytopathic human immunodeficiency virus (HIV) infection (Stevenson et al. 1987). Infection of a human lymphoid cell line with HIV resulted in loss of not only the CD4 marker, which is the receptor for the virus, but also a parallel decline in CD3, CD8 and CD2 surface marker expression. As a consequence, the cell were unable to respond to the infection. The molecular basis and significance of this "multireceptor-downregulation", which is

accompanied by unresponsiveness is not yet known but might have some similarities with the immunosuppressed system.

Expression of the IL-2 receptor did not show the pattern of consistent depression that was found for Thy-1, Lyt 2 and L3T4. The limitation of the present approach based on assaying for the 55kd chain of the IL-2 receptor has already been discussed. Others have also found that the relationship between the amount of IL-2 receptor on cell surface and cell proliferation is not simple or direct (Mills et al. 1985; Smith and Cantrell, 1985).

The model system used here shows that IL-2 plays an essential role in the development of the primary anti-viral CTL response in vivo, and is probably necessary for both T cell proliferation and differentiation. Furthermore, the results suggest that decreased expression of cell surface molecules involved in cell-cell interactions and signal transduction by lymphocytes may explain the impaired responsiveness of CTL.

Chapter 4

Requirements for the development of a secondary anti-viral cytotoxic T cell response <u>in vitro</u>.

4.1 Introduction

One of the hallmarks of an immune response is the phenomenon of specific immunological memory. This is most easily demonstrated by the rapid and strong increase in levels of antibody following re-exposure to antigen. However, cytotoxic T cell memory responses are measured indirectly since it is difficult to detect CTL activity by assay of cells from animals that are re-infected with the same pathogen. It is possible that in many cases the memory T cell response is not activated due to the rapid removal of the inoculum by antibody and also antibody-dependent cell-mediated cytotoxicity (Dunlop and Blanden, 1976).

The presence of primed CTL has been demonstrated following infection with most commonly studied animal viruses e.g. influenza, pox and LCM viruses, by restimulating the in vivo primed cells with antigen for several days in culture (rev. by Zinkernagel and Rosenthal, 1981). This method generates highly potent, specific T cells although it requires the addition of an exogeneous cell population, namely antigen-presenting or stimulator cells, for optimal stimulation. These memory T cells differ from unprimed T cells both quantitatively and qualitatively. They are present at higher frequencies (generally >1/60 000 in naive and approximately 1/1000 in immune spleen) and show evidence of higher affinity for appropriate target cells, (Ryser and MacDonald, 1979; MacDonald et al. 1981 & 1982). The relative resistance of the CTL progeny of primed CTL precursors to inhibition by anti-CD8 mAb is generally regarded as a characteristic of CTL carrying high avidity antigen receptors (MacDonald, 1981 & 1982; Malissen et al. 1982; Moretta et al. 1984; Shimonkewitz et al. 1985).

It has also been proposed that memory T cells maintain increased levels of IL-2 receptors on the cell surface (Lefrancois et al. 1984) although this has not been confirmed by studies which examined expression of the 55 kd chain of the IL-2 receptor with anti-Tac antibody (Siegel et al. 1987). However, memory CTL have been shown to have increased reactivity to IL-2. Re-expression of specific CTL activity has been achieved in the absence of antigen by incubating resting long-term MLR cells with secondary MLC supernatant (Ryser et al. 1978; Wagner and Rollinghoff, 1978; MacDonald et al. 1980). More convincingly it has been shown that T cell clones and also positively selected Lyt 2^+ cells re-express specific cytolytic activity on culture

with recombinant IL-2. In addition it was found that, in the presence of IL-2, only the Lyt-2^+ cells are necessary for the development of cytotoxic activity (Devos et al. 1984; Yoshimoto et al. 1985; Ochoa et al. 1986; Lefrancois et al. 1984). IL-2 induces the expression of the β chain of its own receptor (Reem and Yeh, 1984; Malek and Ashwell, 1985; Smith and Cantrell, 1986). Thus, if memory T cells continuously express the α chain of IL-2 receptor (as proposed recently by Siegel 1987 et al.), IL-2 could then induce the formation of high affinity IL-2 receptors which would mediate signal transduction and lead to the re-activation of these T cells.

It has been found, that activation of T cells, whether through interaction of TCR with antigen-MHC or by binding of mAb to some cell surface proteins results in the turnover of membrane phosphoinositides (PI). As shown in Fig. 4.1, binding of antigen to its receptor results in hydrolysis of PIP_2 by phospholipase C. The products of this hydrolysis are diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). DG activates a Ca^{2+}-dependent enzyme, protein kinase C (PKC) which can phosphorylate a wide range of protein substrates by using ATP as phosphate donor. IP_3, the other product of the PIP_3 hydrolysis releases Ca^{2+}, probably from the endoplasmatic reticulum, which may provide a sufficient concentration of intracellular Ca^{2+} for the activation of PKC which then phosphorylates numerous substrates. In T cells, the substrates for PKC activity include the IL-2 receptor which becomes phosphorylated at Ser 247 on the β chain (Shackelford and Trowbridge, 1984) and the gamma chain of the TCR-T3 complex (Cantrell et al. 1985; Nel et al. 1987; Samelson et al. 1985b & 1987).

The two crucial signals for the stimulation of T cells, namely activation of PKC and increase in intracellular Ca^{2+} concentration can be achieved by the combined effects of phorbol esters (DG analogues) and calcium ionophores. This stimulation is mitogenic for both T and B lymphocytes (KrÖnke et al. 1985; Roifman et al. 1987). Activation of T cells involves a series of cellular and intracellular reactions, which culminate in lymphocyte differentiation, proliferation, lymphokine production and cytolytic activity. The early effects on cells stimulated with phorbol esters and CaI include an increase in intracellular Ca^{2+}-concentration (Imboden et Stobo, 1985), internalization of the TCR-antigen complex (Reinherz et al. 1982; Ando et al. 1985;Pantaleo et al. 1987a; Takada and Engleman, 1987) and expression of the nuclear proto-oncogenes c-fos and c-myc (Krönke et

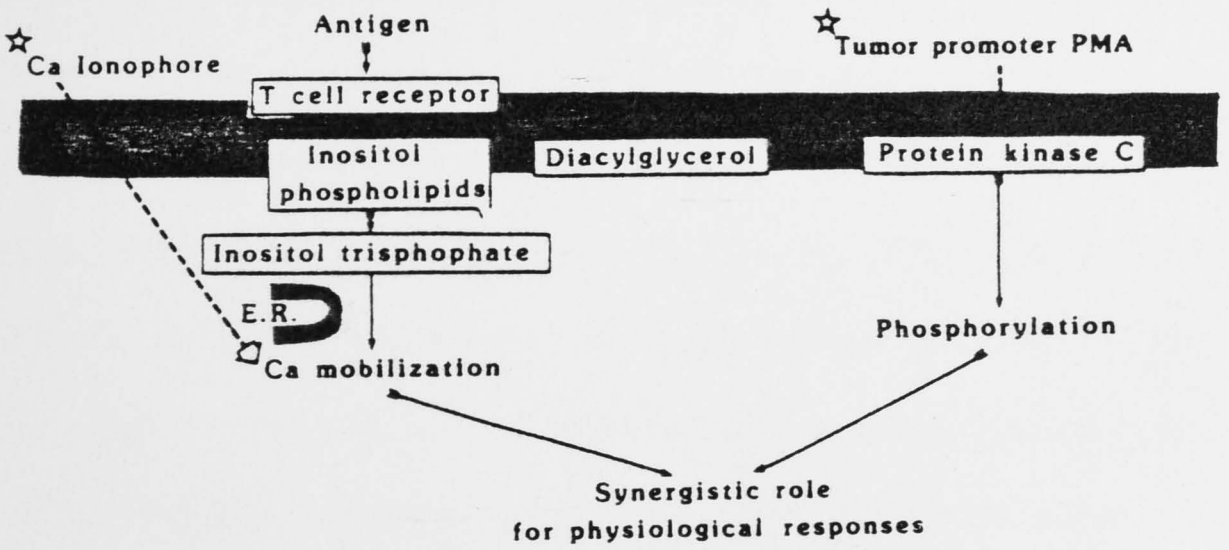


Fig. 4.1 Biochemistry of T cell activation

al. 1985; Reed et al. 1985a; Kumagai et al. 1987). These oncogenes may play an important role in the control of the proliferative responses (Greenberg and Ziff, 1984; Muller et al. 1984).

In this chapter the requirements for activation of resting, in vivo primed LCMV-immune T cells are studied. Re-expression of immune T cell function was analysed at both the bulk and clonal levels. In addition, use of PMA and CaI provided an antigen-independent stimulation protocol, which was used to estimate the frequency of LCMV-specific pCTL in primed mice. Finally, some of the possible applications of this method of T cell activation are discussed.

4.2 Results

4.2.1 Specific stimulation of LCMV-immune spleen cells in vitro.

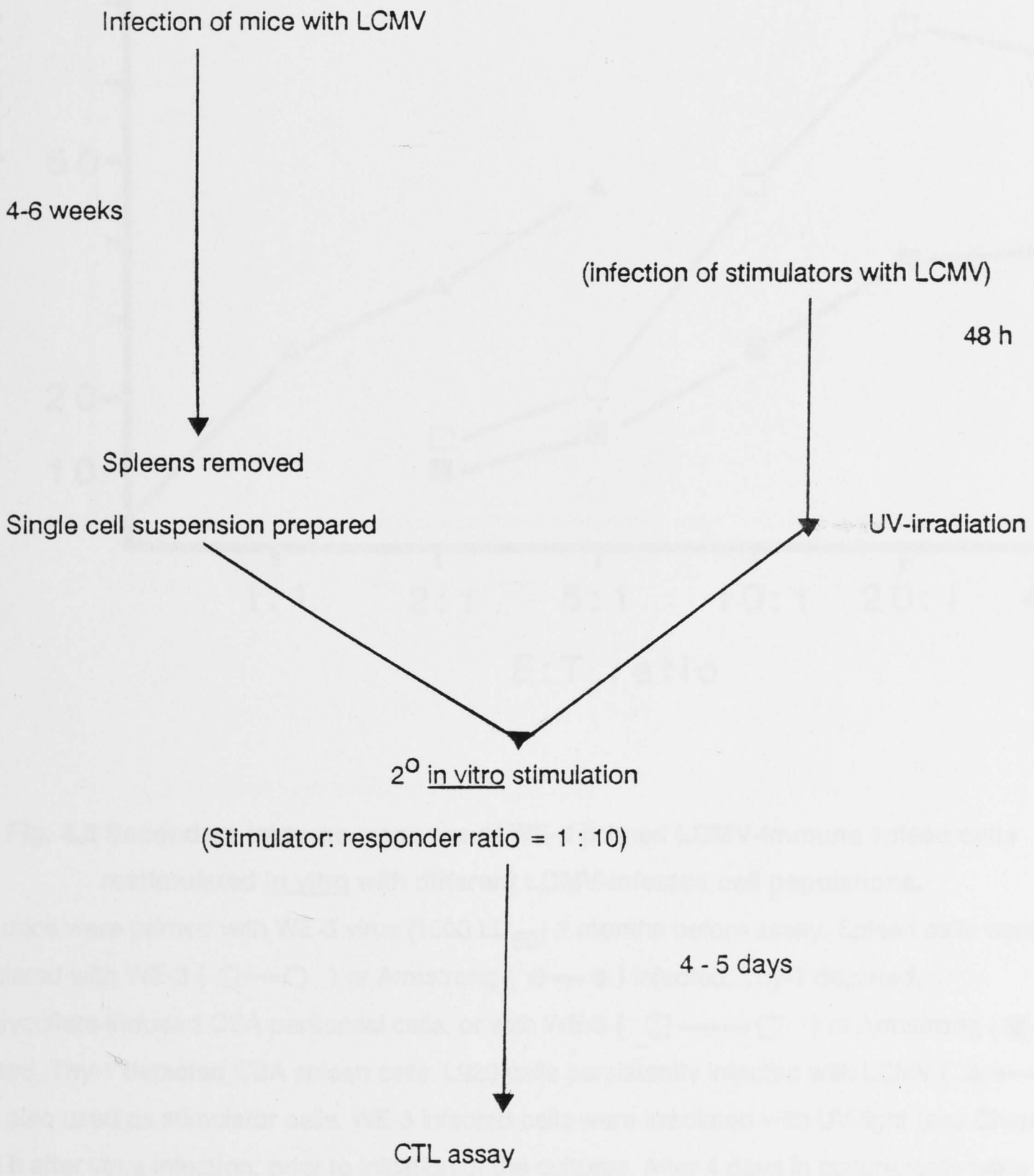
Following the method shown in Fig. 4.2, LCMV-infected peritoneal macrophages, T cell-depleted spleen cells and L929 fibroblast cells were compared for their ability to stimulate the in vitro secondary CTL response of LCMV-immune spleen cells (Fig. 4.3). Cells were also infected with the two most often used strains of LCMV, WE-3 (viscerotropic) and ARM (neurotropic) and compared for their ability to act as stimulators.

The WE-3 strain is cytopathic in vitro, and has, therefore, to be inactivated before adding responder cells to the cultures. As shown on Fig. 4.3, stimulating the WE-3 primed memory cells with WE-3 LCMV infected, UV-irradiated cells resulted in a more effective secondary CTL response than that found following the use of ARM LCMV to infect stimulator cells. This was also true when the animals were primed with the ARM strain of the virus. (Fig. 4.4). Furthermore, based on the results of several experiments, e.g. Fig. 4.3, thioglycollate-induced peritoneal macrophages were more effective stimulators than T cell-depleted spleen cells or persistently infected L929 fibroblasts. In the latter the recovery of effector cells from the culture was especially low (<20%).

The differences in the resulting secondary lytic activity using different strains of viruses and cell types, may be explained by quantitative or qualitative differencies in the expression of MHC molecule-antigen complexes on the surface of antigen presenting cells (APC).

Taken together, in this experimental model, WE-3 infected, thioglycollate-induced peritoneal macrophages were the most effective stimulators of LCMV-immune memory CTL-response. This system was than used routinely.

Fig. 4.2 Protocol for restimulation of LCMV immune T cells <u>in vitro</u>.



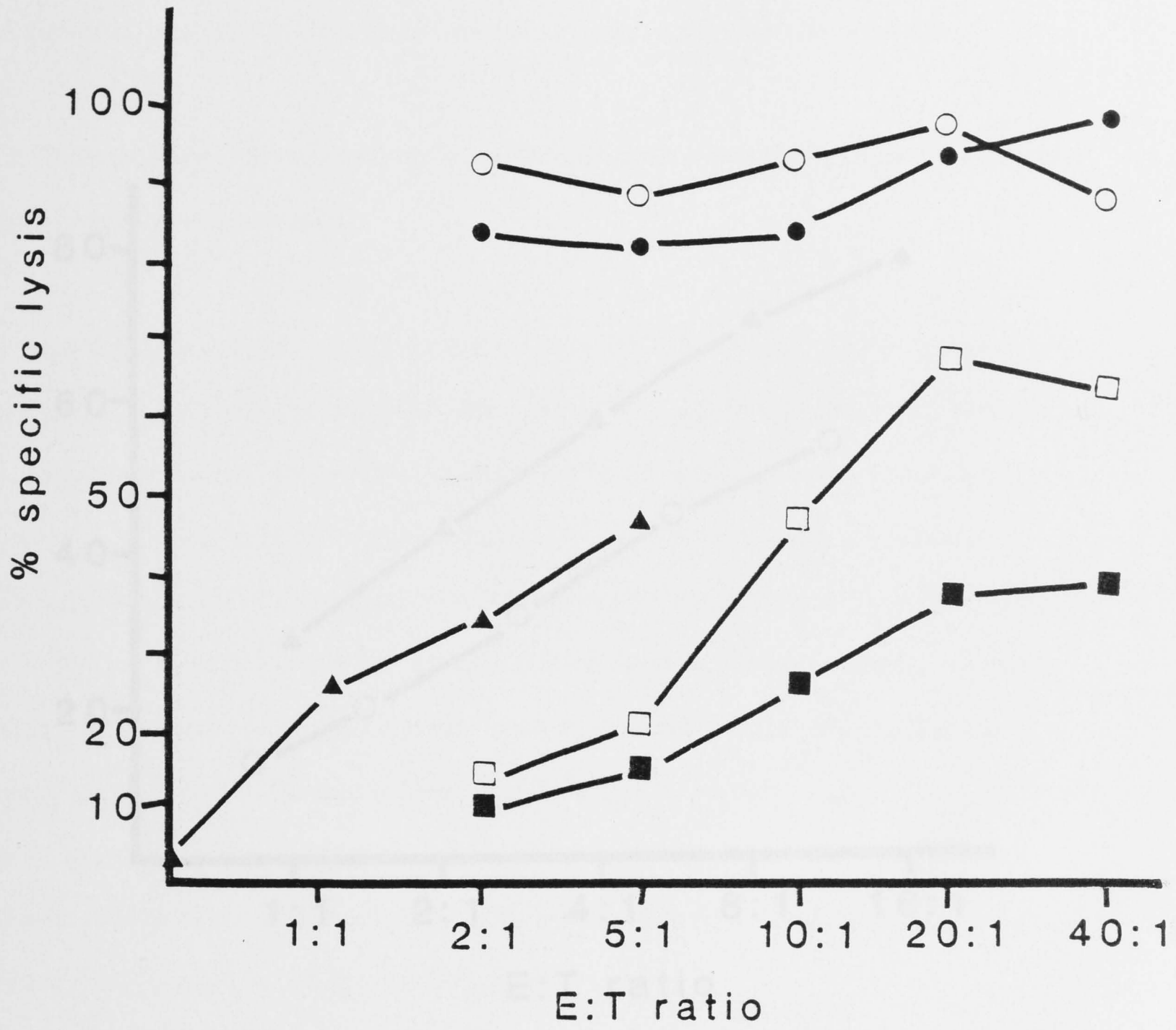


Fig. 4.3 Secondary immune response of WE-3 primed LCMV-immune spleen cells restimulated <u>in vitro</u> with different LCMV-infected cell populations.

CBA mice were primed with WE-3 virus (1000 LD_{50}) 2 months before assay. Spleen cells were stimulated with WE-3 (○—○) or Armstrong (●—●) infected, Thy-1 depleted, thioglycollate-induced CBA peritoneal cells, or with WE-3 (□ —— □) or Armstrong (■— ■) infected, Thy-1 depleted CBA spleen cells. L929 cells persistently infected with LCMV (▲—— ▲) were also used as stimulator cells. WE-3 infected cells were irradiated with UV-light (see Chapter 2) 48 h after virus infection, prior to initiation of the cultures. After 4 days in culture, cells were collected and counted, the recovery was usually less than 25%. Cytotoxic activity of the effectors was measured in a 6 hr Cr-release assay on WE-3 infected CBA macrophage target cells. The mean of triplicate samples is shown, S.E. <5%. Killing of uninfected target cells was the highest in group (○— ○) being 20%, but was less then 8% for the other groups (E:T = 40:1).

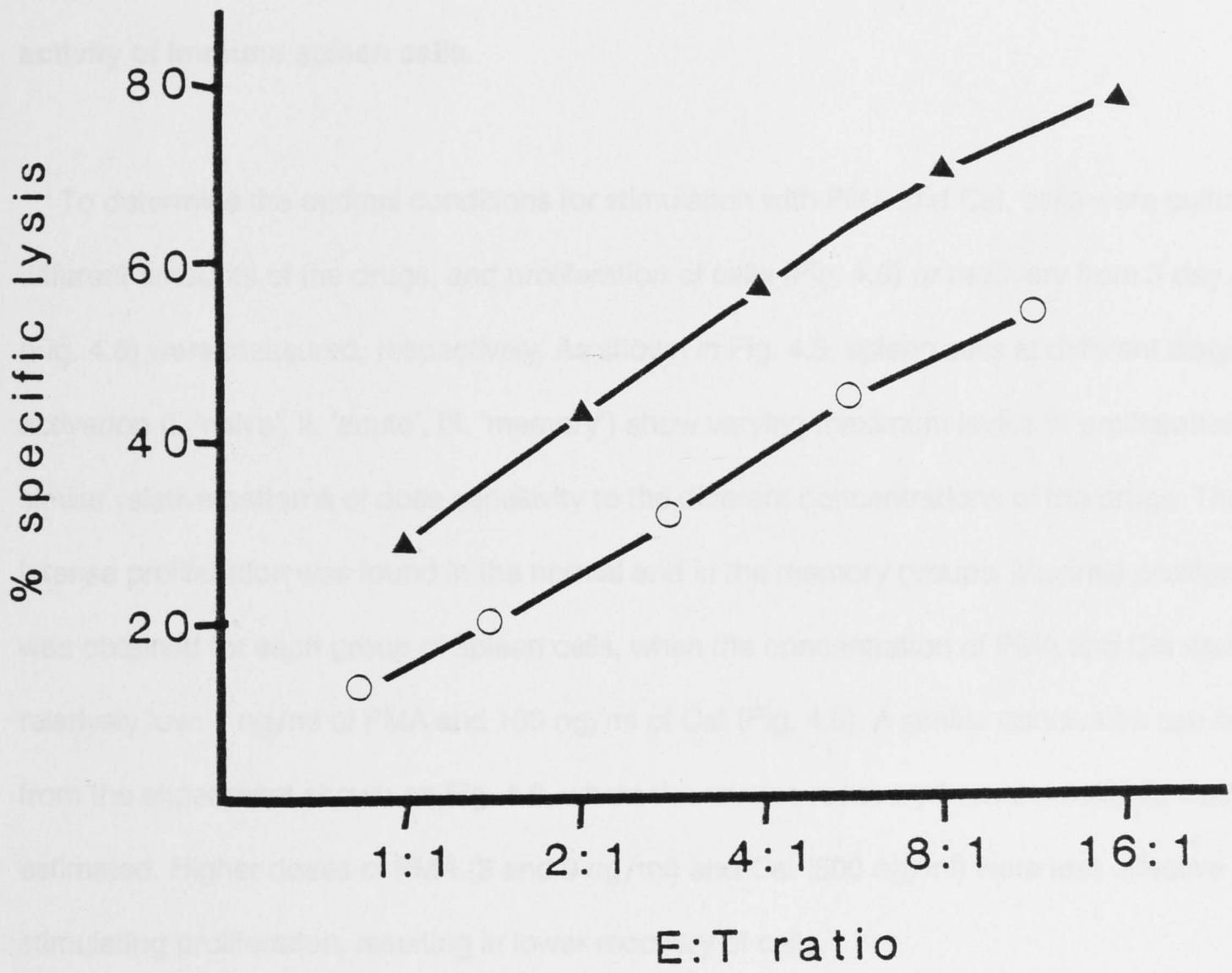


Fig. 4.4 Secondary immune response of LCMV-Armstrong primed spleen cells, restimulated in vitro.

Balb/c mice were primed with Armstrong-strain of LCMV (100 LD_{50}/mouse) 2 months before the assay. Their spleen cells were stimulated with WE-3 (▲——▲) or Armstrong (○—○) infected, Thy-1 depleted, thioglycollate-induced Balb/c peritoneal cells. WE-3 infected cells were irradiated with UV-light before culture. Cytotoxic activity of effectors was tested 5 days later in ^{51}Cr-release assay using LCMV WE-3 infected P815 targets. Mean of triplicate samples is shown, S.E. being less than 1.5%.

4.2.2 Stimulation of LCMV-immune spleen cells in vitro in the absence of antigen.

4.2.2.1 Effect of different concentrations of PMA and CaI on the proliferation and cytotoxic activity of immune spleen cells.

To determine the optimal conditions for stimulation with PMA and CaI, cells were cultured with different amounts of the drugs, and proliferation of cells (Fig. 4.5) or recovery from 3 day cultures (Fig. 4.6) were measured, respectively. As shown in Fig. 4.5, spleen cells at different stages of activation (I. 'naive', II. 'acute', III. 'memory') show varying maximum levels of proliferation but similar relative patterns of dose sensitivity to the different concentrations of the drugs. The most intense proliferation was found in the normal and in the memory groups. Maximal proliferation was obtained for each group of spleen cells, when the concentration of PMA and CaI was relatively low: 1 ng/ml of PMA and 100 ng/ml of CaI (Fig. 4.5). A similar conclusion can be drawn from the experiment shown on Fig. 4.6, where the relative recovery from the cultures was estimated. Higher doses of PMA (3 and 9 ng/ml) and CaI (500 ng/ml) were less effective in stimulating proliferation, resulting in lower recovery of cells.

To check whether the optimum levels of proliferation and specific lytic activity could be achieved by the same combination of doses of the drugs, groups C and F (Fig. 4.5), representing the highest and the lowest proliferation induced by PMA and CaI were also tested for their specific lytic activity in a ^{51}Cr-release assay (Table 4.1). According to the results of the proliferation assay, higher concentrations of PMA and CaI were less effective at inducing specific lytic activity by memory CTL (Groups A and B; C and D, Table 4.1). High concentrations also inhibited the lytic activity of acutely primed LCMV-immune spleen cells (Groups E and F). Importantly, however, neither low nor high combinations of the drugs induced cytolytic activity by unprimed spleen cells (Groups G and H).

4.2.2.2 The effect of IL-2 on PMA and CaI stimulated cultures.

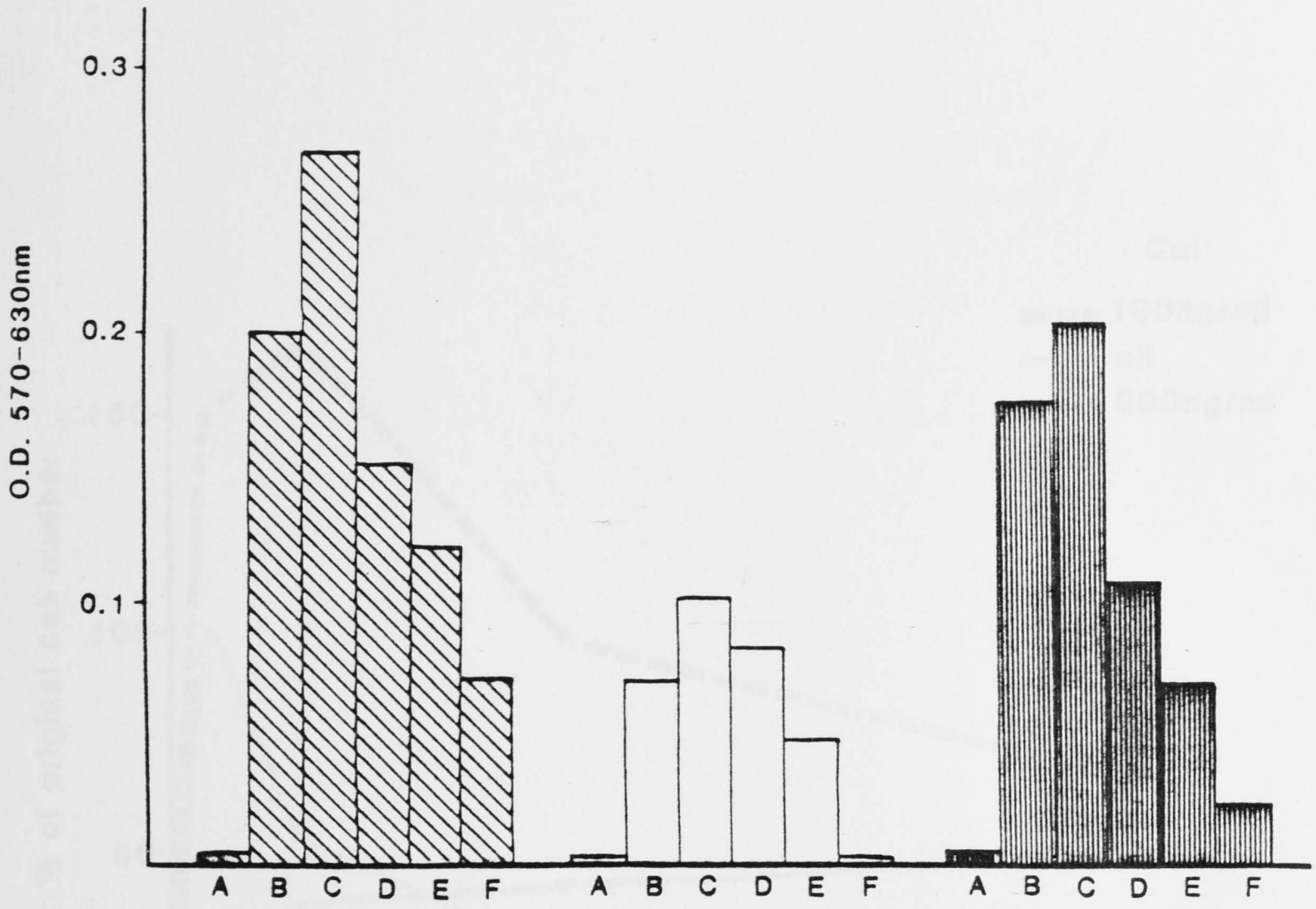


Fig. 4.5 Proliferation of spleen cells upon PMA and Cal stimulation.

Spleen cells from normal B6 (▨), acute, LCMV immune (6 day) B6 (□) and memory LCMV-immune (B6 x CBA)F_1 (6 weeks) (▥) mice were cultured in the absence (A) or in the presence of 0.5 ng/ml PMA & 50 ng/ml Cal (B), 1 ng/ml PMA & 100 ng/ml Cal (C), 1.5 ng/ml PMA & 150 ng/ml Cal (D), 2 ng/ml PMA & 200 ng/ml Cal (E), 3 ng/ml PMA & 300 ng/ml Cal (F) for 3 days in the presence of 5 % EL-4 sn. Proliferation of 10^4 cells/well was measured by MTT assay. Mean of triplicate samples is shown.

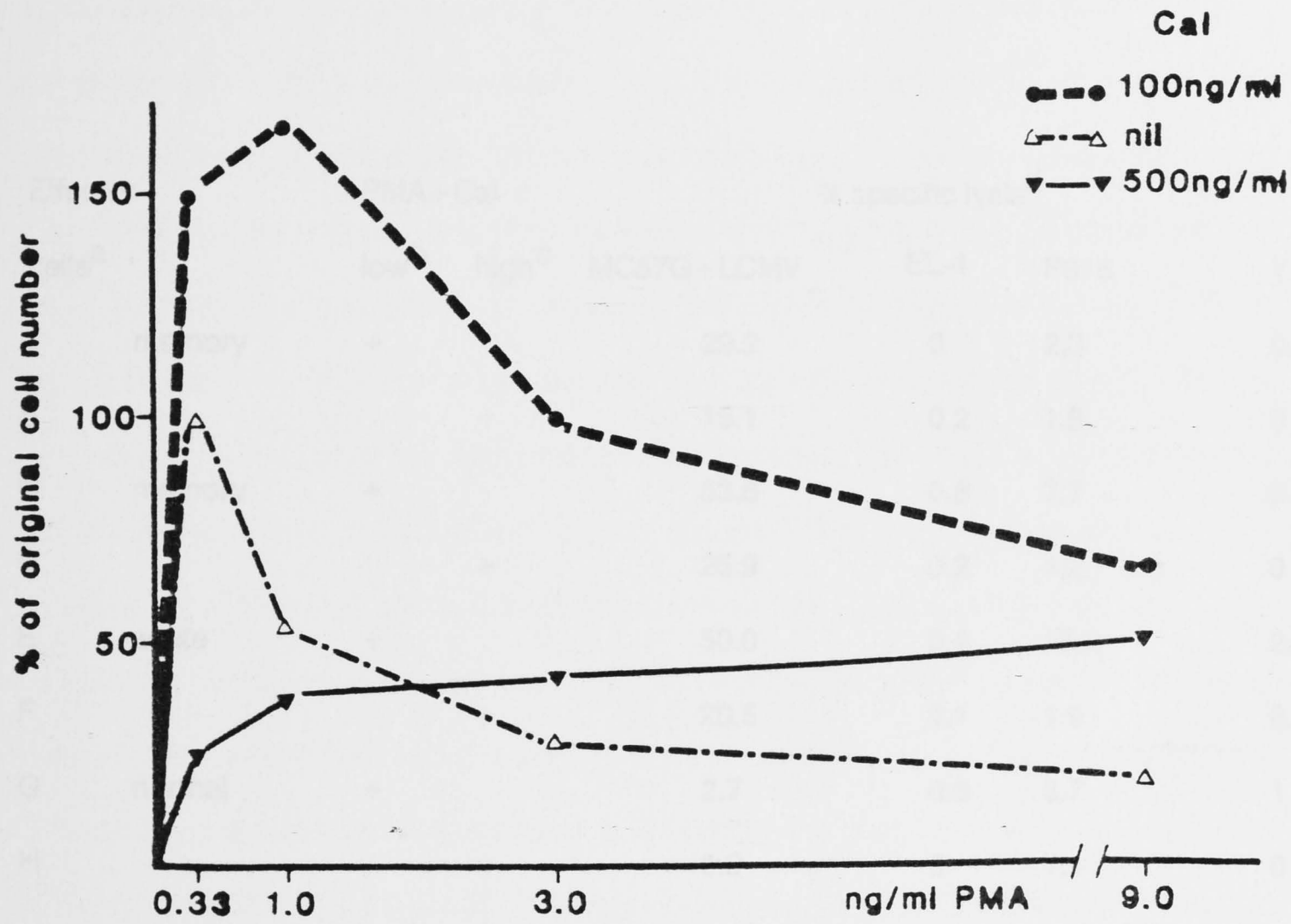


Fig. 4.6 Cell recovery upon PMA and Cal stimulation.

10^6 LCMV primed Balb/c spleen cells/well were cultured for 3 days in 24 well trays in the presence of PMA, Cal and 5 % EL-4 sn. The cells were collected and counted at the end of incubation, percent recovery per group is shown.

Table 4.1 Effect of high and low concentration of PMA and Cal on cytotoxic activity and specificity of the lysis.

Effector cells[a]		PMA + Cal low[b]	PMA + Cal high[c]	% specific lysis[d] MC57G + LCMV	EL-4	P815	YAC
A	memory	+		29.2	0	2.3	0.3
B			+	15.1	0.2	1.6	0
C	memory	+		53.6	0.8	7.7	0.4
D			+	25.9	0.2	4.2	0
E	acute	+		50.0	0.4	13.2	2.4
F			+	20.5	0.1	1.9	0.3
G	normal	+		2.7	0.8	3.7	1.7
H			+	0.9	0	1.1	0

a) spleen cells were harvested from B6 mice, infected with LCMV 6 weeks before the assay (Groups A & B), from (CBA x B6)F_1 mice, infected with LCMV 6 weeks before the assay (Groups C & D), from B6 mice, 7 days after infection (groups E & F), and from normal B6 mice (groups G & H).

b) effector cells were cultured in the presence of 1 ng/mlPMA and 100 ng/ml Cal for 3 days.

c) effector cells were cultured in the presence of 3ng/ml PMA and 300 ng/ml Cal for 3 days

d) Effector:target ratio is 10 : 1. Spontaneous lysis of targets is less than 26%, mean of triplicate samples is shown, S.E. is less than 3.6%.

The necessity for, and effect of, adding IL-2 to PMA and Cal stimulated cultures was studied. Proliferation of spleen cells in the presence of 5 U/ml highly purified recombinant IL-2 was more intense in the initial period (48h) of culture for both memory and normal cells (Table 4.2, groups C,D and G,H). Later, (4 days in culture) this difference was less marked, probably due to sufficiently high levels of endogeneously produced IL-2. Treatment with IL-2 alone resulted in much lower levels of proliferation (Table 4.2, Groups B and F). IL-2 itself, without the presence of the original priming antigen, can induce re-expression of CTL function, as summarized in Table 4.3. The lytic activity induced by IL-2 seemed to depend on the amount of IL-2 added (Table 4.3, groups B and C), but the level of non-specific lysis also increased in these groups. However, when the same amount of IL-2 was added in the presence of PMA and Cal, re-expression of lytic activity proved to be highly specific (Table 4.3, groups D, G and J). The recovery of cells was 5-10 times higher than that after IL-2 stimulation, or even from cultures stimulated with virus (200% vs 30-50%). Exogeneous IL-2 also increased both the level of cytolytic activity and the number of cells recovered from PMA and Cal stimulated cultures (Table 4.3 I and J; Table 4.4 A and B).

PMA and Cal separately are inefficient at stimulating the re-expression of the lytic activity of immune T cells (Table 4.4 C and D). They also cause only very minor, if any, proliferation of cells as measured by % recovery from the cultures (Table 4.4 C and D vs F). However, when exogeneous IL-2 was provided to these cultures, lytic activity was detected. This seemed to be more specific than that induced by the same amount of IL-2 alone, but less intense than that caused by the application of the drugs together (Table 4.4 H and I vs E).

4.2.2.3 The effect of the incubation time of PMA and Cal stimulated cultures.

The length of incubation was also studied to determine its effect to the magnitude and specificity of killing. As shown in Table 4.3 Group J, specific lytic activity appears as early as 24 h after initiating the cultures. A longer incubation does not seem to result in more effective CTL activity from high density cultures (Fig. 4.7). It is hard to interpret such experiments, since either

Table 4.2 Effect of exogeneous IL-2 on PMA and Cal induced proliferation.

Group	Effector	Treatment	[^{3}H]-thymidine uptake (cpm)	
			48 h	96 h
A	normal	nil	433 ± 73	ND[a]
B		IL-2	904 ± 122	ND
C		PMA + Cal	6956 ± 2442	ND
D		PMA + Cal + IL-2	19561 ± 1541	105265 ± 3249
E	memory	nil	411 ± 24	1153 ± 170
F		IL-2	1293 ± 152	4859 ± 1215
G		PMA + Cal	22730 ± 7027	109679 ± 3326
H		PMA + Cal + IL-2	37193 ± 7178	115435 ± 4224

Effector cells are B6 spleen cells from uninfected (normal) or LCMV infected (memory) mice (injected 5 weeks before the assay)

5 U/ml rec. IL-2 was added in groups B,D,F & H.

a) Not done.

Table 4.3 Effect of exogeneous IL-2 on the PMA and CaI induced lysis.

Exp.	Effectors	In vitro treatment	% specific lysis a	b	c	recovery d	
1 A	B6 spleen	nil	9	0	0	0	16%
B		5 U/ml IL-2	42	16	9	ND	26%
C		10 U/ml IL-2	45	21	17	8	21%
D		PMA+CaI+IL-2	33	0	0	1	210%
2 E	CBA x B6	nil	9	1	1		29%
F		10 U/ml IL-2	34	33	34		43%
G		PMA+CaI+IL-2	27	8	7		195%
3 H	CBA x B6	5 U/ml IL-2	69	27			
I		PMA+CaI	47	1			
J		PMA+CaI+IL-2	69	12			
K (unprimed)		PMA+CaI+IL-2	16	2			

Mice were primed with WE-3 LCMV 4-7 weeks before the assay and their spleen cells were cultured for 3 days (Exp 1 & 2) and for 24 h (in Exp. 3), respectively, in the absence (A,E) or in the presence of added rec.IL-2 (B,C,F and H groups). In groups D, G, J and H, 1ng/ml PMA, 100 ng/ml CaI and 10 U/ml IL-2 (rec.) was added.

The targets are: a) MC57G+LCMV, b) MC57G uninf., In Exp.1 c)P815, d)YAC. E:T ratio was 20:1.

In Exp.2 the target c) is KD2SV+LCMV. E:T ratio was 40:1.In Exp.3 E:T ratio was 80:1.

Table 4.4 Stimulation of secondary CTL response by PMA and Cal applied separately.

Exp.	Group	Stimulation			% recovery	% specific lysis		
		PMA	Cal	IL-2		MC57G*	MC57G	KD2SV*
1	A	+	+	+	195	26.9	8.3	7.5
	B	+	+	-	159	26.1	7.8	8.6
	C	+	-	-	45	9.2	3.2	5.2
	D	-	+	-	43	10.4	5.4	0.6
	E	-	-	+	47	33.8	34.0	33.0
	F	-	-	-	39	9.3	0.8	1.0
2								P815
	G	+	+	+	100	30.7	0	0
	H	+	-	+	22	21.4	0	ND
	I	-	+	+	22	15.3	0	ND
	J	-	-	-	16	9.4	0	0

* target cells infected with LCMV

Exp.1. (CBA x B6)F_1 mice were primed 10 weeks before the assay. For in vitro stimulation of spleen cells 1 ng/ml PMA, 100 ng/ml Cal and 10 U/ml rec.IL-2 was added. Cells were incubated for 3 days, and cytotoxicity measured at 40:1 effector:target ratio.

Exp.2. Spleen cells from (B6 x Balb/c)F_1 mice, primed with LCMV 10 weeks before the assay were stimulated as above. E:T ratio 20:1.

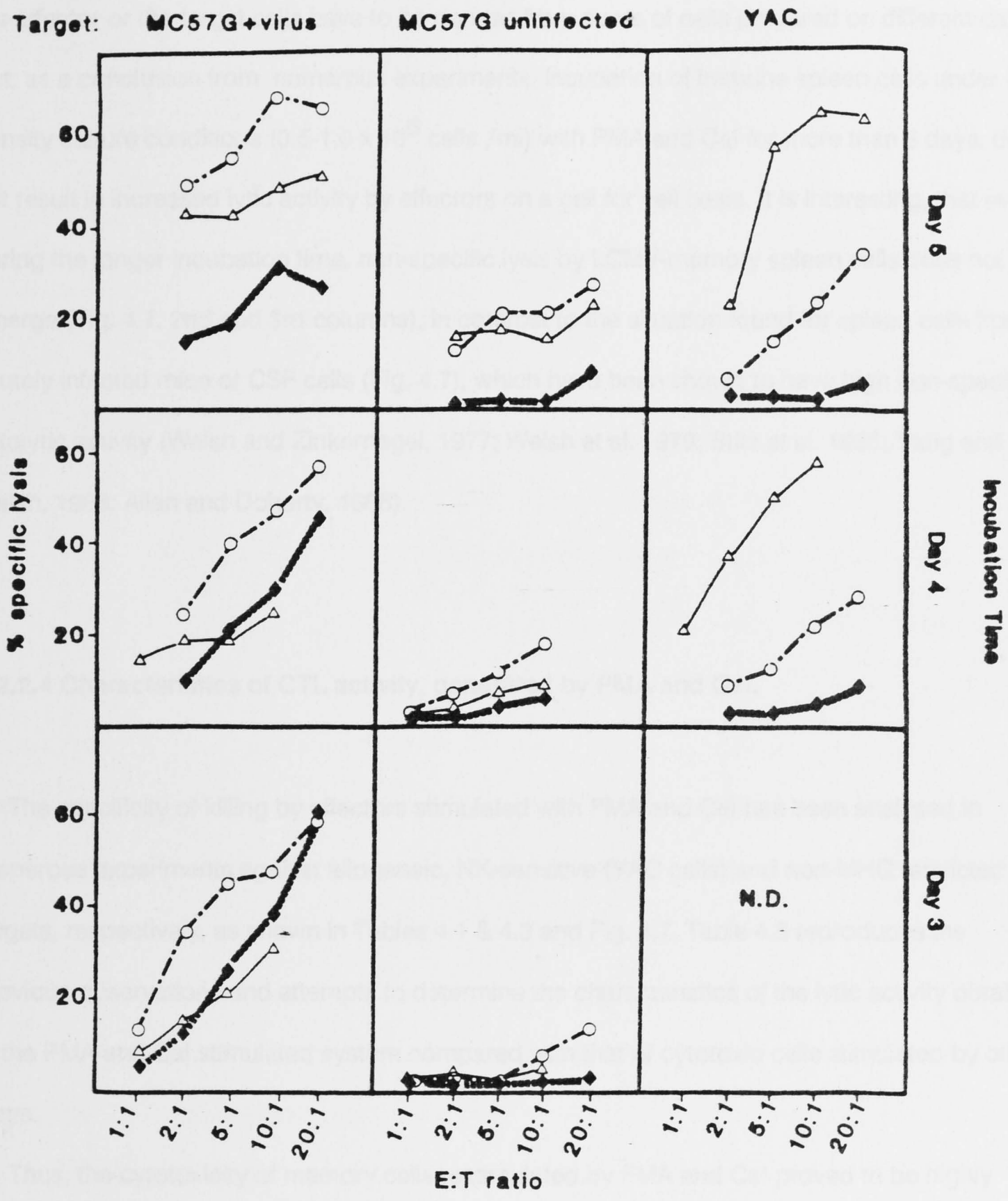


Fig. 4.7 Effect of incubation time with PMA and Cal on the specificity of killing by LCMV-immune CTL.

LCMV primed 6 day immune (O——O) and 6 weeks immune (◆''''◆) spleen cells, or cerebrospinal fluid (CSF) cells from i.c. injected (CBA x B6)F_1 mice (△—— △) were cultured for 3 (A), 4 (B) or 5 (C) days in the presence of 100 ng/ml Cal and 1 ng/ml PMA. Lytic activity on different targets was measured in ^{51}Cr-release assay.

the effector or the target cells have to be derived from pools of cells prepared on different days. But, as a conclusion from numerous experiments, incubation of immune spleen cells under high density culture conditions (0.5-1.0 x 10^6 cells /ml) with PMA and CaI for more than 3 days, does not result in increased lytic activity by effectors on a cell for cell basis. It is interesting, that even during the longer incubation time, non-specific lysis by LCMV-memory spleen cells does not emerge (Fig. 4.7, 2nd and 3rd columns), in contrast to the situation found for spleen cells from acutely infected mice or CSF cells (Fig. 4.7), which have been shown to have high non-specific cytolytic activity (Welsh and Zinkernagel, 1977; Welsh et al. 1979; Stitz et al. 1985; Yang and Welsh, 1986; Allan and Doherty, 1986).

4.2.2.4 Characteristics of CTL activity, generated by PMA and CaI.

The specificity of killing by effectors stimulated with PMA and CaI has been analysed in numerous experiments against allogeneic, NK-sensitive (YAC cells) and non-MHC restricted targets, respectively, as shown in Tables 4.1 & 4.3 and Fig. 4.7. Table 4.5 reproduces the previous observations and attempts to determine the characteristics of the lytic activity obtained in the PMA and CaI stimulated system compared with that of cytotoxic cells stimulated by other ways.

Thus, the cytotoxicity of memory cells restimulated by PMA and CaI proved to be highly specific: the procedure did not cause any significant allo- or other non-specific killing by these cells. Furthermore, the lytic pattern is distinct from that of NK or LAK cell mediated lysis, in that both systems show high levels of killing of YAC cells and allogeneic target cells (Table 4.5, Group C vs A & B). The lytic cells induced by IL-2 seemed to differ from those with NK activity. Cells collected on day 3 after infection, when there is high activity against YAC cells, did not show lectin-dependent cytotoxic (LDC) activity (Table 4.5, group A, last column), while quite potent LDC activity was found for the other two groups (B and C): this presumably reflects that lymphokine-activated killer (LAK) effectors and activated CTL (B), or activated CTL alone (C), are responsible for lysis.

Table 4.5 Specificity of PMA and Cal stimulated killing.

Effectors	Treatment	% specific lysis[a]				
		MC57G+LCMV (b)	P815 (d)	YAC (a)	L929 (k)	P815+PHA (d)
A)CBA(k) 3d	nil	41	12	73	16	4
B)B6xBalb/c (bd)(6 weeks)	IL-2[b]	50	25	69	21	100
C)B6xBalb/c (bd)(6 weeks)	PMA+Cal+IL-2	39	5	7	2	60

a) Spontaneous lysis of targets was between 14.6 and 30%. E:T ratio = A) 60:1, B) 40:1

b) 100 U/ml EL-4 sn, 3 days incubation

Group C: 1ng/ml PMA, 100 ng/ml Cal, 5 U/ml EL-4 sn, 3 days incub.

Taken together, the results of these experiments suggest that stimulation of memory spleen cells with PMA and Cal can result in the appearance of a functionally homogeneous CTL population, which does not have the characteristics of LAK or NK cells.

4.2.3 Studies on PMA and Cal stimulated secondary CTL response at the clonal level.

Memory spleen cells were cultured under limiting dilution conditions in the presence of 1 ng/ml PMA, 100 ng/ml Cal and IL-2. As for bulk cultures, these concentrations provided the optimal stimulus for proliferation of cells in low density cultures. The frequency of LCMV-specific CTLp determined under these conditions was greater than 1:1000 for primed spleen cells, tested with LCMV-infected targets, while effectors lytic for uninfected targets were not detectable at the 1:30 000 level (Fig. 4.8). In addition, LCMV-specific CTLp were present at a higher frequency in spleen than in lymph node (Table 4.6, A and C) and LCMV-immune cells were also detectable in the thymus of mice primed with the virus 6 weeks previously (Table 4.6, Group D). In the latter case, immature thymocytes were depleted with J11d antibody and complement treatment (see Chapter 2, section 7.3). The same depletion resulted in some enrichment of pCTL frequency measured in spleen (Table 4.6, Group B).

The specificity of these CTL clones for virus-infected, but not for uninfected syngeneic or allogeneic targets was confirmed in a further experiment (Fig. 4.9, panels A and B). Furthermore, the culture conditions did not lead to the generation of natural killer cells (Panel C, Fig. 4.9). The distribution of lectin-dependent cytotoxicity paralleled that of the LCMV-specific CTL (Panels D and A, Fig. 4.9). Culture of LCMV memory spleen cells in the presence of PMA, Cal and low levels of exogeneous IL-2 thus leads to the specific stimulation of virus-reactive, MHC-restricted CTL clones.

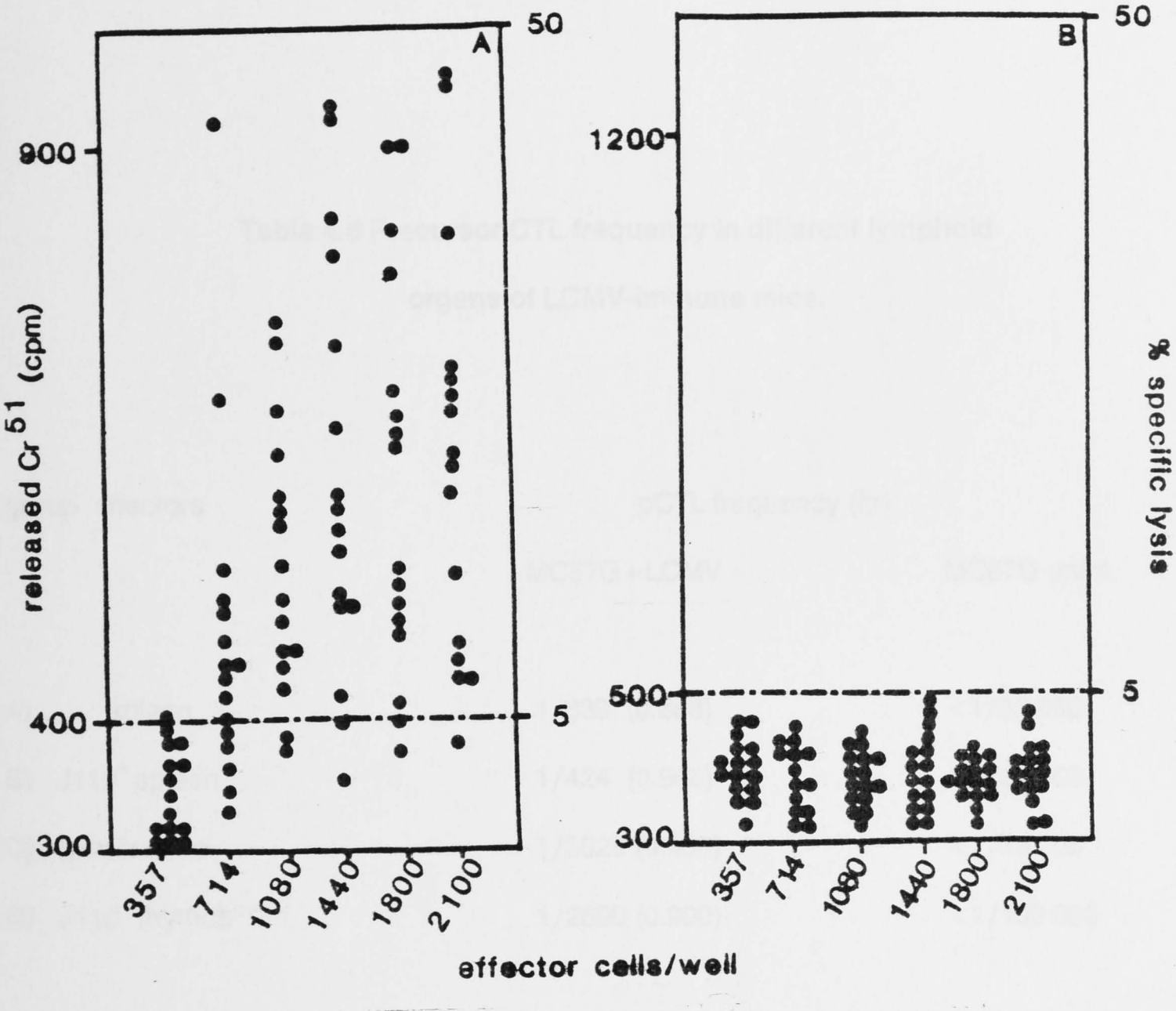


Fig. 4.8 Stimulation of secondary immune response with PMA and Cal at the clonal level.

Spleen cells from (CBA x B6)F_1 mice were cultured for 8 days under LDA conditions (see 2.) in the presence of 1 ng/ml PMA, 100 ng/ml Cal and saturating amounts of IL-2. Cells from individual wells were resuspended, split 1:2 and their lytic activity checked on LCMV-infected (A) or uninfected (B) L929 target cells. Released ^{51}Cr (cpm) from 16 individual wells per group is shown. The dotted line represents 5% specific lysis of targets calculated by the formula in Chapter 2, section 13.

Table 4.6 Precursor CTL frequency in different lymphoid organs of LCMV-immune mice.

group	effectors	pCTL frequency (f;r)	
		MC57G + LCMV	MC57G uninf.
A)	spleen	1/639 (0.888)	<1/30 000
B)	J11d⁻ spleen	1/424 (0.968)	<1/30 000
C)	lymph node	1/3028 (0.900)	<1/55 000
D)	J11d⁻ thymus	1/2690 (0.990)	<1/100 000

B6 mice were primed with LCMV 5-7 weeks previously. The cells were incubated as described at Fig. 4.8 and in Chapter 2, section 9.2.

Wells were considered positive if specific killing was above 5%. pCTL frequency was calculated as described in Chapter 2, section 13.

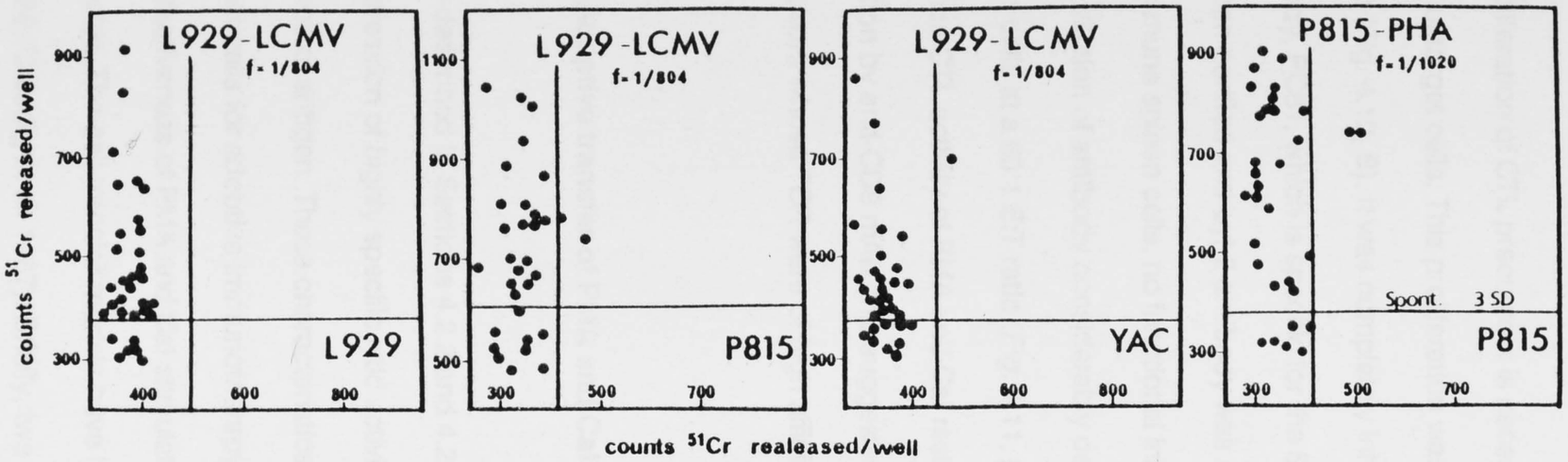


Fig. 4.9 Specificity of the PMA and Cal stimulated clonalsecondary CTL response.

B6 spleen cells were cultured (10^3 per well) as described in Fig. 4.8. After 8 days in culture, individual wells were split 1:2 and their lytic activity tested against a panel of target cells. Dots represent the released ^{51}Cr (cpm) induced by effector cells from individulal wells.

4.2.4. Inhibition of PMA and CaI stimulated proliferation and lytic activity of CTL clones by monoclonal antibodies

Proliferation of CTL precursors is necessary during LDA experiments to achieve detectable killing of target cells. This proliferation was reduced considerably when insufficient IL-2 was added (Fig. 4.10, B). It was completely inhibited by co-culturing the cells with purified monoclonal antibody, PC61, which is specific for the 55 kd β chain of the IL-2 receptor (Fig. 4.10, D).

When purified anti-Lyt 2 antibody was added to individual LDA wells, originally containing 5 x 10^3 immune spleen cells, no functional inhibition of CTL activity occured (Fig. 4.11, panel B). The same dilution of antibody considerably decreased the lytic activity of 'acute' (6 d LCMV-immune) spleen cells at a 60:1 E:T ratio (Fig. 4.11, panel A).

Since CTL activity of PMA and CaI restimulated LCMV-immune CTL was relatively resistant to inhibition by anti-CD8 mAb, this suggested that these effector CTL derived from 'primed' precursors whose TCR were of high affinity for antigen.

4.2.5 Adoptive transfer of PMA and CaI treated cells.

As described in Sections 4.2.2 and 4.2.3, stimulation of T cells with PMA and CaI results in the re-expression of highly specific lytic activity and intensive proliferation of precursors in the absence of antigen. These characteristics could be very useful for obtaining sufficient numbers of effector cells for adoptive immunotherapy. Therefore, experiments were designed to determine the effectiveness of PMA and CaI stimulated LCMV-immune cells in adoptive cell transfer protocols. The cell transfer models have been discussed in greater detail elsewhere (Doherty et al. 1986; Ceredig et al. 1987). Briefly, two experimental models were used, using immunosuppressed and unsuppressed recipients, respectively, as summarized in Fig. 4.12.

The results of two experiments using the Cy-immunosuppressed model are shown in Table 4.7. In these experiments the extravasation of effector T cells into the CSF can be considered to be a function of the CTL mediated immune response, since specific interaction between virus-

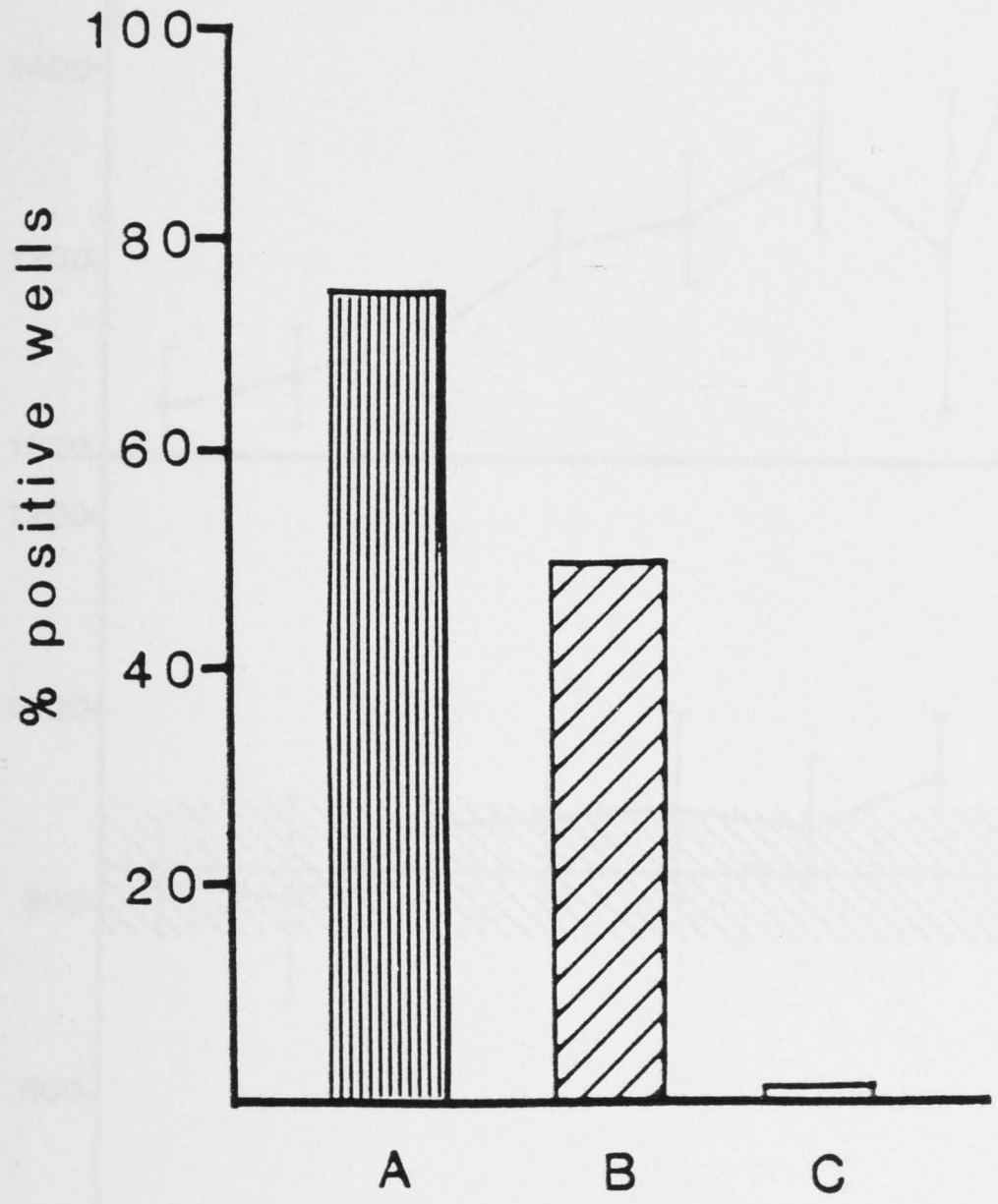


Fig. 4.10 Effect of anti-IL-2 receptor antibody on the proliferation of clones of LCMV-immune CTL stimulated by PMA and Cal.

Spleen cells (800/well) from B6 mice primed 6 weeks previously with LCMV were cultured in the presence of 1 ng/ml PMA, 100 ng/ml Cal and saturating amounts of IL-2 (group A). Group B wells were supplemented with IL-2 (20 μl/well) only once at 48 h after initiation of the culture. In group C, cultures were set up as usual (as group A) and 50 μg/ml purified PC61 antibody was added. Clonal expansion was estimated 7 days later by light microscopy. For each group 96 wells were tested.

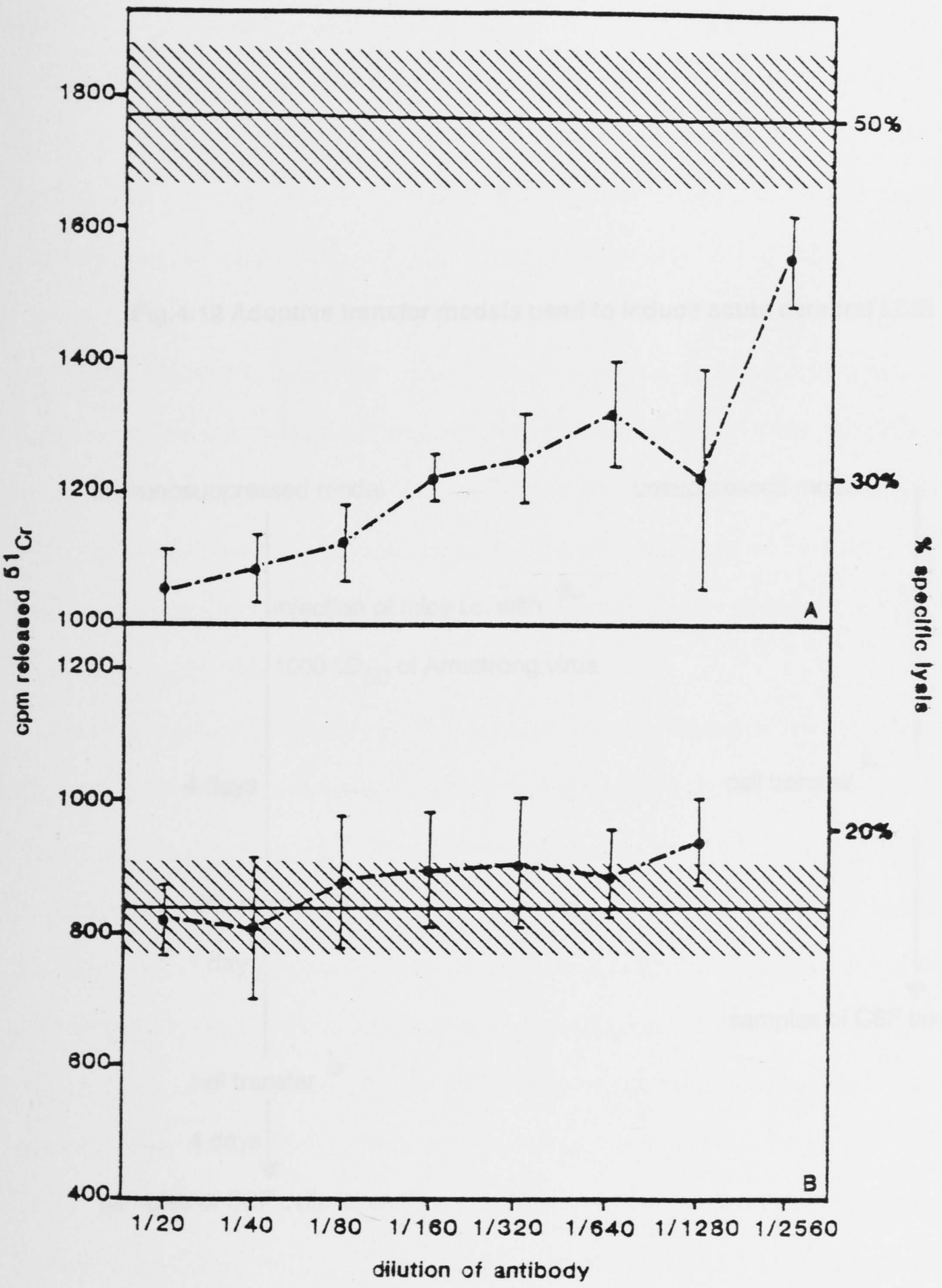
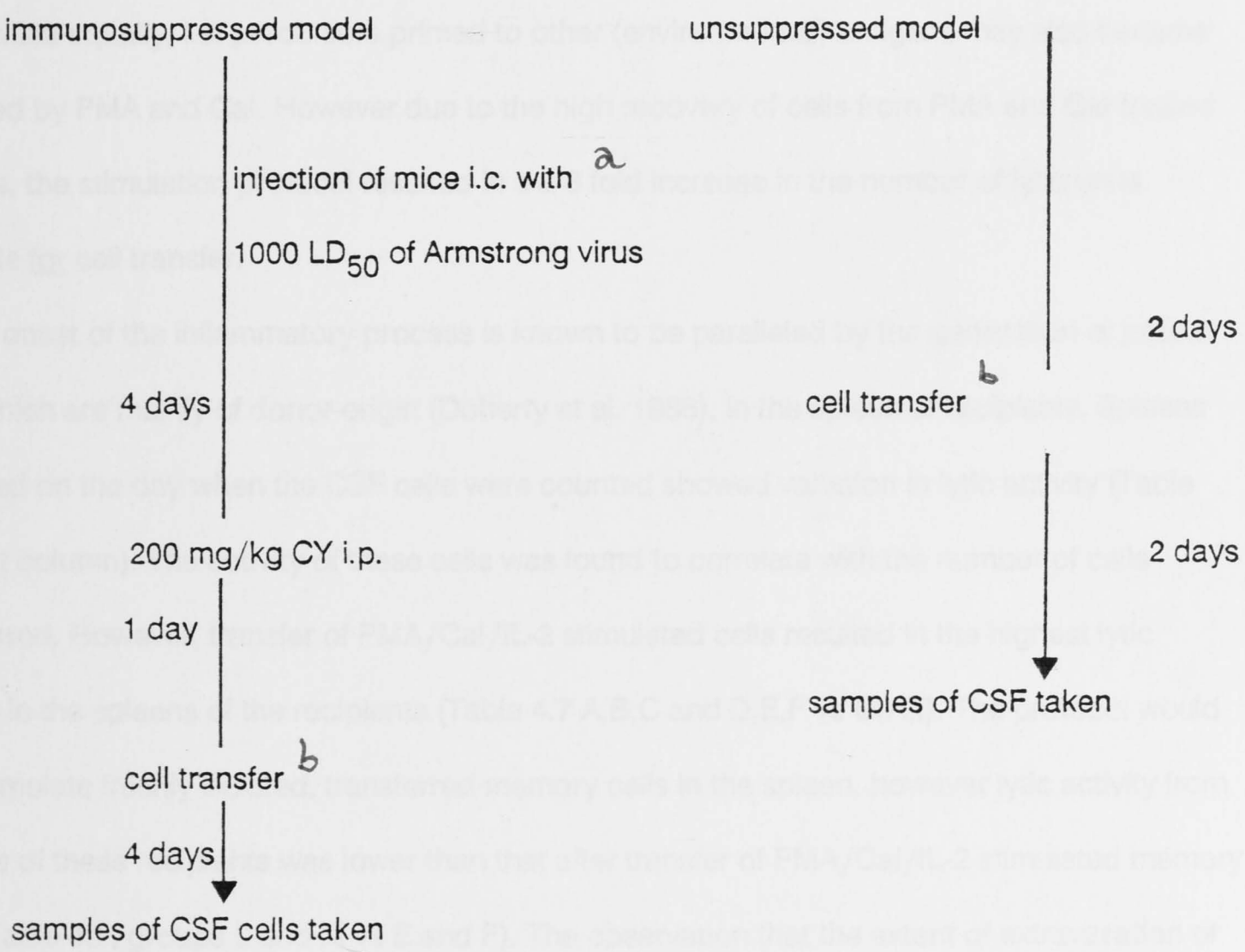


Fig. 4.11 Inhibition of primary but not PMA stimulated secondary CTL response by anti-Lyt-2 antibody.

Cytotoxicity of spleen cells of acutely infected (6 day immune) (A) or 10 week immune mice stimulated with PMA and Cal (B) as described at the previous figures, was measured in the absence (striped area) or in the presence of purified 53.6.1 (anti-Lyt-2 antibody) on LCMV-infected MC57G target cells. E/T ratio for A is 60:1. For B, 5×10^3 cells were cultured per well in vitro for 8 days. The mean and SD of quadruplicate samples are shown.

Fig.4.12 Adoptive transfer models used to induce acute cerebral LCM



a Recipient mice for both the immunosuppressed and unsuppressed models

b Transfer of immune spleen cells from donor mice primed 7 or 8 days previously

immune Lyt-2$^+$ cells and virus-infected cells must occur (Doherty and Allan, 1986; Baezinger et al. 1986). The level of extravasation correlated with the number of transferred cells (Table 4.7, groups A-C and D-F). Transfer of PMA/Cal/IL-2 stimulated cells (groups A-F) resulted in a considerably higher cell number in the CSF, although the meningitis was not as severe as that following the transfer of spleen cells from donors infected with LCMV 7 days previously (groups D,E,F vs H,I,J). This protocol probably allowed sufficient time for reactivation in vivo of memory cells in groups which had not been treated with PMA and Cal (groups L and M vs E and F). Therefore the PMA+Cal+IL-2 treatment may have stimulated T cell clones with different specificities equally, i.e. precursors primed to other (environmental) antigens may also became activated by PMA and Cal. However due to the high recovery of cells from PMA and Cal treated cultures, the stimulation protocol resulted in a 2-3 fold increase in the number of lytic units available for cell transfer.

The onset of the inflammatory process is known to be paralleled by the generation of potent CTL, which are mainly of donor origin (Doherty et al. 1986), in the spleen of recipients. Spleens collected on the day when the CSF cells were counted showed variation in lytic activity (Table 4.7, last column). The activity of these cells was found to correlate with the number of cells transferred. However, transfer of PMA/Cal/IL-2 stimulated cells resulted in the highest lytic activity in the spleens of the recipients (Table 4.7 A,B,C and D,E,F vs G,H,I). The protocol would also stimulate freshly isolated, transferred memory cells in the spleen, however lytic activity from spleens of these recipients was lower than that after transfer of PMA/Cal/IL-2 stimulated memory cells (Table 4.7, groups L and M vs E and F). The observation that the extent of extravasation of transferred cells into the CSF did not directly correlate with their lytic activity in the spleens of recipients 4 days after cell transfer could be explained by differencies in homing properties of the intravenously injected cells.

In the unsuppressed model, the experiments were manipulated to minimize the time available for in vivo activation of transferred cells and also to avoid any limitations due to homing, by injecting the donor cells i.c.. The results of these experiments are summarized in Table 4.8. The use of the unsuppressed model and the i.v. route of cell transfer resulted in only a slight increase in the number of infiltrating cells in each group except when acute spleen cells were injected

Table 4.7 Adoptive cell transfer of PMA/Cal/IL-2 stimulated LCMV-immune spleen cells: immunosuppressed model.

Exp.	Group	p.i.[a]	In vitro culture	Cells transferred ($\times 10^7$)	$\log_{10}$ cells /μl of CSF	% spec.lysis
1	A	8 wk	+	0.4	2.87 (31x)[c]	71.3
	B		+	0.8	3.25 (74x)	82.4
	C		+	1.6	3.62 (174x)	84.8
2	D	11 wk	+	2.0	3.81 (282x)	85.1
	E		+	1.0	2.94 (38x)	80.4
	F		+	0.5	2.37 (9.3x)	65.6
	G	Nil	+	2.0	1.18 (<1x)	16.0
	H	1 wk	-	2.0	4.40(1096x)	45.7
	I		-	1.0	4.08 (525x)	50.4
	J		-	0.5	3.27 (81x)	45.5
	K	Nil	-	2.0	1.61 (1.8x)	0.4
	L	11 wk	-	1.0	3.10 (55x)	68.1
	M		-	0.5	2.37 (10x)	46.3

a) B6 mice primed with 1000 LD_{50} WE-3 virus i.p. Time in weeks between priming and assay is shown. p.i.: post infection.

b) Donor cells were cultured in the presence of I ng/ml PMA, 100 ng/ml Cal and 10% (v/v) EL-4 sn for 3 days, except groups H,I,J,K,L,M where freshly isolated spleen cells were transferred i.v.

c) Increase above background (injected with medium) is shown in brackets.

d) Lysis by recipient spleens (containing donor-derived cells) on LCMV-infected MC57G target cells. E:T ratio is 50:1 in Exp.1 and 100:1 in Exp.2.

Table 4.8 Adoptive cell transfer of PMA/Cal/IL-2 stimulated LCMV-immune spleen cells: unsuppressed model.

Exp.	Group	Priming[a] of donors	PMA (h)	$\log_{10}$ cells/μl CSF i.v.		i.c.
1	A	17 weeks	-	3.60	(1.7x)	4.08 (3.7x)
	B		24	3.32	(<1.0x)	4.15 (4.4x)
	C		48	3.43	(1.1x)	4.22 (5.1x)
	D		72	3.54	(1.4x)	4.33 (6.6x)
	E	7 days	-	4.76	(24x)	4.16 (4.5x)
	F	unprimed	72	3.38	(1.0x)	3.56 (1.0x)
2	G	4 weeks	-	2.60	(<1.0x)	4.41 (17.0)
	H		48	3.43	(<1.0x)	4.54 (22.9)
	I		96	3.31	(<1.0x)	4.14 (9.1x)
	J	7 days	-	4.46	(10.2x)	4.17 (9.8x)
	K	unprimed	48	3.45	(1.0x)	3.58 (2.5x)

1a) B6 mice were primed and stimulated as described in Table 4.7. Number of transferred cells: 2 x 10^7 i.v.; 1 x 10^7 i.c. in Exp.1. and 6 x 10^6 i.c. in Exp.2.

b) Increase above background (injected with medium) is shown in brackets.

(Table 4.8, groups E and J). However i.c. injection proved to be effective and in this case the transfer of PMA/Cal/IL-2 stimulated memory cells resulted in severe meningitis (Table 4.8, groups B,C,D,H). Incubating the cells with PMA /Cal/IL-2 for different periods before cell transfer gave results somewhat similar to those for the kinetics of development of CTL activity (Fig. 4.7): stimulation with the drugs for more than 3 days did not increase the effectiveness of these cells on adoptive transfer. (Groups B,C,D,H vs. I).

The phenotype of cells after different lengths of stimulation with PMA/Cal/IL-2 was also analysed and will be discussed in greater detail in Chapter 5. Expression of the Mel 14 marker, which has been suggested to be involved in the in vivo homing of lymphocytes (Dailey et al. 1985) decreased during in vitro stimulation, and was relatively higher on acute or freshly isolated memory lymphocytes (Table 5.2). Whether this observation is relevant to the finding that i.v. injected, PMA+Cal stimulated cells are less able to accumulate on the site of infection needs more detailed analysis.

4.2.6 Use of PMA and Cal to study T cell non-responsiveness.

Mice infected with LCMV at birth develop a persisitent infection which is thought to result from immunological tolerance. LCMV-specific CTL and DTH cannot be detected (Lehmann-Grube, 1983) and this does not appear to be explained by deletion of LCMV-reactive T cell clones since anti-LCMV IgG antibody is produced (Oldstone, 1975; Oldstone et al. 1980; Ahmed and Oldstone, 1985). At about 1% of peripheral $CD4^+/CD8^-$ lymphocytes is infected with the virus during persistent infection (Ahmed and Oldstone, 1987). It was of interest therefore to see if the absence of LCMV-specific $CD4^+/CD8^-$ cells results in 'specific tolerance' to LCMV among $CD8^+/CD4^-$ cells and whether this could be overcome by growing the cells in PMA and Cal in the presence of IL-2.

Balb/c mice were injected i.p. with the Armstrong strain of virus within 24h of birth. Spleen cells were collected 6 weeks later and stimulated with LCMV-infected stimulator cells or with PMA/Cal/IL-2, respectively. The lytic activity of these cells and also cells from LCMV-memory normal mice was determined against target cells infected with WE-3 or Armstrong virus strains.

Spleen cells from mice persistently infected with LCMV could not be stimulated in vitro with virus-infected cells (Table 4.9.I). Furthermore they did not express any CTL activity (above background) upon stimulation with PMA and CaI in the presence of IL-2, even when very high concentration of PMA and CaI were used. (Table 4.9.II).

Thus, the unresponsiveness of CTL is probably not due to a failure in the TCR-mediated activation of the phosphoinositide breakdown pathway, because the alternative method of activation with PMA and CaI did not restore the cytotoxic T cell response against LCMV.

Table 4.9.I. Specific stimulation of spleen cells from LCMV-carrier mice.

Group	Eff.[a]	Stim.[b]	% specific lysis[c]			
			KD2SV + WE-3	KD2SV + Arm	MC57G + WE-3	MC57G
A	memory	WE-3	69	51	100	21
B	memory	Arm.	57	46	86	ND
C	carrier	WE-3	12	15	ND	0
D	carrier	Arm.	6	14	16	0

a) memory: (B6 x Balb/c)F_1 mice primed with 1000 LD_{50} of WE-3 virus 4 weeks before the assay. Carrier: Balb/c mice primed i.p. with Arm. virus within 24 h of birth.

b) Specific restimulation of effectors was carried out with syngeneic, T cell-depleted spleen cells infected with the virus strain shown in the table, as described in 2.8, for 3 days.

c) E:T ratio = 50:1

Table 4.9.II.Stimulation of spleen cells from LCMV carrier mice with PMA/Cal/IL-2.

Group	PMA	Cal[d]	% specific lysis[e]					
	(ng/ml)		KD2SV+WE-3		KD2SV+Arm		MC57G+WE-3	
			C	M	C	M	C	M
E	1	100	7	34	12	30	2	52
F	1	300	0	20	7	24	0	38
G	1	900	10	15	10	31	2	41
H	3	100	1	27	2	22	2	49
I	3	300	2	20	7	19	3	36
J	3	900	4	16	10	26	10	38
K	9	100	2	24	8	16	6	39
L	9	300	5	13	7	21	9	21
M	9	900	13	15	7	11	12	16

d) Incubation for 3 days in the presence of 10 % (v/v) EI-4 sn also added

e) Effector:target ratio is 25:1. C:carrier; M:memory (same as in Table 4.9.I.a). Spontaneous lysis of target cells was 34, 38 and 24%, respectively. Mean of triplicate samples is shown, S.E. being less than 5%.

4.3 Discussion

Phorbol esters together with calcium ionophore have been found to be a convenient method of activating populations of T and B cells. This procedure has allowed a detailed analysis of the biochemical pathway involved in T cell activation. Prior to this study there has been no report on the specificity of T cells activated by these drugs. This chapter analysed the potential of PMA and Cal treatment to reactivate and grow anti-viral memory T cells while retaining antigen-specificity.

Specific restimulation with virus of in vivo primed LCMV immune spleen cells usually resulted in slightly higher cytotoxic activity than that found following stimulation with PMA and Cal. However the latter T cells seemed to be more virus-specific than populations stimulated directly with virus, or with larger amounts of IL-2. High concentrations of IL-2 stimulate both antigen-specific T cells expressing high affinity IL-2 receptors and also other cells identified as lymphokine activated killer cells (LAK) (Yron et al. 1980; Lotze et al. 1981). LAK cells share some characteristics with 'classical' CTL and NK cells, but it is likely that each arises from a different population of precursors (Grimm et al. 1983; Andriole et al. 1985). Recent studies have suggested that they also differ with respect to serological markers, kinetics of induction and sensitivity to irradiation: while LAK effector cells are Thy-1$^+$, LAK precursors are Thy-1$^-$,Ia$^-$, non-T, non-B lymphocytes (Rosenberg and Lotze, 1986), they require 3-7 days for generation, while NK cells only 1-2 days. LAK effector cells kill NK-resistant target cells, and can be induced from spleen cells of beige mice which do not have normal NK activity (Merluzzi et al. 1986). LAK cells are also resistant to treatment with anti-asialo GM_1.

Thus, stimulation of memory CTL with PMA and Cal resulted in intense proliferation and potent lytic activity, with a high degree of specificity of killing by unseparated, in vivo primed spleen cells.

Both allo-immune (Truneh et al., 1985; Isakov and Altman, 1985) and, as shown in this chapter, virus-immune cells can be triggered with PMA and Cal to generate cytotoxic effector function only after in vivo priming. Thus requirements for induction of secondary T cell responses to viruses and alloantigens seem to be similar. Moreover it seems that the potent alloreactive response of unprimed T cells following stimulation with antigen does not simply reflect recent

exposure to environmental antigens such as viruses, as these cells are not triggered to cytotoxic effector function by PMA and Cal.

The frequency of CTL precursors was found to be about 1/800 spleen cells, as determined by the antigen-independent method. This is in accord with pCTL frequencies found by other groups for vaccinia (see Chapters 3 and 6), influenza (Owen et al. 1982), and LCMV - determined by restimulation with virus - (Moskophidis et al. 1987) and indicates that CTL clones specific for the different antigenic determinants of the virus are probably represented at a distribution comparable to that found following specific restimulation.

The fact that only primed CTL appear to be activated by PMA and Cal to kill appropiate target cells seems to be sufficient to explain the effect of these drugs in the induction of the secondary anti-viral and alloreactive responses. The possibility that antigen persists on APC of primed mice thereby accounting for the apparent specificity of the response cannot be excluded. Studies on the clearance of LCM virus from the spleens or peripheral blood of acutely infected animals have shown that infectious virus can no longer be detected 8 days after infection (rev. by Buchmeier et al. 1980) but some cells, e.g. macrophages or dendritic cells are able to retain antigen for long periods and stimulate memory B cell responses (Mandel et al. 1980; Tew et al. 1980; Klaus et al. 1980). However they represent a very small population (< 1%) of spleen cells, thus it seems to be less evident that they play a crucial role in the development of the secondary T cell response stimulated by PMA and Cal. The finding that J11d$^-$ spleen cells and thymocytes can also be stimulated in the absence of antigen also support the conclusion that a contribution by APC is unlikely during stimulation of memory cells by PMA and Cal.

Murine lymphocytic choriomeningitis (LCM) is the classsical model of T cell-mediated immunopathology in virus infections. Experimental models have been exploited in which the CTL activity of adoptively-transferred immune spleen cells - measured <u>in vitro</u> - correlated with their capacity to induce symptoms of LCM in virus infected (i.c.), CY-suppressed (Doherty et al. 1986; Allan and Doherty, 1985) or unsuppressed mice (Ceredig et al. 1987). The fatal neurological disease induced in the recipients by adoptively transferred immune spleen cells probably results from acute damage to functional cells in the central nervous system by virus-immune Lyt 2$^+$ lymphocytes (Allan and Doherty, 1985; Doherty et al. 1986; Dixon et al. 1987.).

PMA and Cal stimulated LCMV immune spleen cells show similar virus-specific cytotoxicity as day 7 LCMV infected acute spleen cells. However, their ability to mediate inflammation in the CNS of LCMV infected, immunosuppressed mice remained less than that of acute, in vivo primed spleen cells or in some experiments even less than that of memory spleen cells not stimulated in vitro before the cell-transfer. The relatively long time (3 days) between cell transfer and evaluation of inflammation could be sufficient to allow the in vivo restimulation of some of the transferred memory cells. This delay could account for the in vivo activity of memory spleen cells not restimulated in vitro before cell transfer

The relatively poor ability of PMA + Cal stimulated cells to mediate inflammation in the CSF could be explained by: (1) Immunosuppression with 200 mg/kg CY probably does not eliminate all CTL precursors from the host animal (see in Chapter 3). The apparent absence of host cells in CSF as detected by FMF (Ceredig et al. 1986) could be due to depressed expression of the Thy.1 antigen following the immunosuppression as described in Chapter 3. Spleen cells from acutely infected mice, but not PMA and Cal stimulated cells may contain or produce higher amount of factor(s) necessary to restore the development of CTL in the immunocompromised host. (2) Spleen cells stimulated in vitro have altered migration and homing characteristics, as shown previously for cloned T cells (Dailey et al. 1985), therefore PMA and Cal - stimulated cells may not show normal recirculation patterns. The results of i.c. cell transfer into unsuppressed mice seem to favour this but shows that PMA+Cal stimulated memory cells are able to induce severe inflammation in the CNS if introduced directly.

Mice infected with LCMV in utero, neonatally or as immunocompromised adults become persistently infected with the virus.(Traub, 1938; rev. by Lehman-Grube, 1983; Buchmeier et al. 1980; Oldstone et al. 1986). Persistant viral infections are medically significant, - an estimated 200 million people are persistently infected with hepatitis B virus (Marion & Robinson, 1983) - and they can cause a number of chronic diseases (Wolinsky & Johnson, 1980). A variety of viruses are known to cause persistent infections in man. The classic model of viral persistence is infection of mice with LCMV. Although it has provided significant information about carrier status, the question of the mechanism of suppression of cell-mediated immunity and its role in persistance is still unresolved. Carrier status can be characterized by life-long production of

infectious virus, decreased expression of viral glycoproteins, complete lack or very low levels of CTL activity but unimpaired virus-specific B cell function (Buchmeier et al. 1980). Ahmed and Oldstone (1985) have shown that genetically different viral variants are responsible for the persistence of the virus and the suppression of the CTL response. The nature of this suppression can be explained by lymphotropic variants infecting and killing LCMV-specific CTL, or causing selective functional inactivation (Oldstone et al. 1982). Other possible explanations are: inappropriate presentation of the virus by infected T cells (Fink et al. 1983); inhibition of differentiation of CTL precursors (Rammense et al. 1982) or the presence of high concentrations of antigen in the environment of the responding CTL (Lamb et al. 1983; Matis et al. 1983; Ceredig et al. 1986). In the experiments shown in this chapter, the possibility that the presence of excess virus was in some way blocking signal transduction to CTLp, or memory CTL, was investigated. The finding - that PMA+Cal+IL-2 treatment did not lead to the appearance of LCMV-specific CTL activity in spleen populations of LCMV carrier mice - could either mean that this population has been deleted, or that the suppression of function does not reflect a failure in the signal transduction of CTL that could be overcome by exposure to these drugs. Similarly, neonatally induced tolerance to alloantigen was not abrogated by this method of stimulation (see Chapter 6).

The stimulation protocol has numerous other possible applications. Koizumi et al. (1986) successfully stimulated T cells from MLR/l mice, which carry an autoimmune disease that can be characterized by low surface antigen expression and IL-2 defects. xposure of spleen cells from this strain to phorbol ester and Cal resulted in significantly increased IL-2R expression, IL-2 production and de novo DNA synthesis Miller (1986) also reported that phorbol ester and Cal had restorative effects on immunodeficiency in vitro due to aging.

A procedure for the specific induction of tumor-reactive CTLp, in the absence of any precise knowledge of the nature of the tumor antigen, could also prove very useful. Therapy based on the selective expansion of specifically sensitized T cells in this way may have advantages over protocols depending on non-specific activation of lymphocytes with high concentrations of IL-2 (Mule et al. 1985; Loveland et al. 1986; rev. by Rosenberg and Lotze, 1986).

However, since PMA cannot be metabolized, its effect is virtually irreversible (Kraft and Anderson, 1983). It would be worth considering the use of non-tumor-promoting activators of PKC, e.g. bryostatins (Trenn et al. 1988) or synthetic diacylglycerol (DiC8) which can substitute for the effect of PMA (Manger et al. 1987). The latter cause a transient translocation of PKC which starts to disappear after about 30 min, so its effect might mimic the physiologic activation of PKC more closely.

Taken together, the experiments discussed in this chapter showed that low concentrations of PMA and Cal stimulate the re-expression of specific lytic activity by in vivo primed, resting virus-immune T cells. The reactivated cells killed virus-infected target cells with high specificity, and at similar frequency to those reactivated with virus- infected stimulator cells. Spleen cells treated by PMA and Cal were effective in adoptive cell-transfer experiments, however T cell unresponsiveness in persistently infected mice cannot be overcome by stimulating the cells with these drugs.

Chapter 5

Phenotypic and functional characterization of in vivo primed LCMV-immune T cell subsets

5.1 Introduction

The previous chapter dealt with the stimulation of unseparated spleen cells from virus primed mice using a combination of PMA and CaI stimulation, a treatment which directly triggers, in the absence of antigen, the signal transduction pathway of T cells. This chapter considers whether the same signals can activate different T cell subsets and uses this antigen-independent activation to examine the role of these subsets in the development of the secondary anti-viral response of selected T cell populations.

Phenotypic identification of memory CTL was also attempted. Most investigations which have searched for stable expression of growth factor receptors (Lowenthal et al. 1985; Trowbridge and Omari, 1981) or other cell surface antigens (Clement et al. 1984; Damle et al. 1985; Haynes et al. 1981; Cotner et al. 1983) selectively on memory T cells have failed. Recently however in vitro stimulated human T cells have been shown to express the Ta_1 activation marker (Hafler et al. 1986) and 4F2 glycoprotein (Soumalainen, 1986). Furthermore, the Ta_1^+ subset was found to be enriched for T cells reacting to mumps and tetanus toxoid (Hafler et al. 1986) in individuals sensitized to these antigens. It was found that the Ly 24 molecule appears on the surface of in vitro activated T cells (Lynch et al. 1987), therefore in vivo generated anti-viral memory CTL were examined for the presence of Ly 24.

5.2 Results

5.2.1 Phenotypic analysis of normal and memory spleen cells.

Using single colour FMF, the level of expression of IL-2R, Lyt2 and L3T4 antigens was examined on spleen cells freshly isolated from normal and from LCMV-immune memory mice (Table 5.1). The ratio of $Lyt2^+$ and $L3T4^+$ cells was similar in memory and in naive mice, whereas the proportion of cells carrying the Ly 24 antigen was higher by about 10%, in each of two experiments, from virus - primed in comparison to 'naive' animals. Very low IL-2R (55 kd chain) expression was detected on spleen cells from both groups. Thus, immunization with LCMV does not seem to result in a significant phenotypic shift of T cell markers on splenic lymphoid cells 8-10 weeks after priming although an increase in the number of cells expressing the Ly 24 antigen appears to have occurred.

Immediately following restimulation of spleen cells from LCMV-immune mice in vitro an apparent decrease in the number of cells expressing Thy-1, Lyt 2, L3T4 antigens occured. (Table 5.2 A vs B and D vs F). In addition, there appeared to be a decrease in the number of $T3^+$ cells and an increase in the expression of the IL-2 receptor. By 48-72 hr, expression of these markers had gradually increased (Table 5.2, 48 hr:G; 72 hr:C). In vitro stimulation also resulted in the appearance of the Ly 24 antigen on the surface of T cells (Table 5.2, 6th column) as described originally by Lynch et al. (1987). However the expression of the Mel 14 marker decreased markedly 24 h after the PMA treatment and remained low thereafter (last column, Table 5.2).

5.2.2 The role of T cell subsets in the generation of the secondary CTL response.

The role of $L3T4^+$ and $Lyt\ 2^+$ cells in the generation of a secondary anti-viral CTL response following stimulation with PMA and Cal was studied using negatively selected cell populations. Freshly isolated spleen cells from LCMV-immune mice were treated twice with monoclonal antibodies against either Lyt 2 or L3T4 plus complement, respectively. The efficacy of the

Table 5.1 Phenotypic analysis of normal and memory spleen cells

	% positive cells[b]			
cells[a]	Lyt 2	L3T4	PC61	Ly 24
LCMV-primed	24.3	26.9	0.8	28.5
normal	19.0	26.2	1.2	18.6

a) Spleen cells of (CBAxB6)F_1 normal or LCMV-immune mice, primed 6 weeks before assay.

b) Cells were stained with biotinylated mAb to Lyt-2 or L3T4, followed by avidin-FITC or non-biotinylated anti-IL-2R (PC61) or anti-Ly 24 mAb followed by sheep anti-rat-FITC, respectively. % positive cells refers to % cells staining above the corresponding control samples stained with either avidin-FITC or sheep anti-rat FITC alone. Results are representative of two experiments.

Table 5.2 Phenotypic analysis of memory spleen cells[a] following stimulation with PMA and Cal.

		treatm.	% positive cells[c]						
	Gp.	(hr)[b]	Thy1	T3	Lyt2	L3T4	PC61	Ly24	Mel14
Exp.1	A	0	59.3	35.6	31.6	32.7	25.4	40.5	36.8
	B	24	28.4	19.0	13.5	23.9	38.8	61.0	7.3
	C	72	76.4	57.3	33.0	45.4	47.4	90.5	4.8
Exp.2	D	0	63.7		27.1				
	E	10	61.9		19.3				
	F	24	35.0		13.3				
	G	48	81.9		50.8				

a) B6 mice, primed with LCMV 10 weeks before the assay.

b) Incubation time with 1 ng/ml PMA, 100 ng/ml Cal and 2 % (v/v) EL-4 sn.

c) The cells were stained with monoclonal antibodies (biotinylated in case of T3) and with sheep anti-rat-FITC (avidin-FITC for T3).

depletion was analysed by FMF and is shown on the first panel of Fig. 5.1. Stimulation with PMA and Cal for 3 days did not alter the ratio of Lyt 2^+ to L3T4$^+$ cells in the unseparated spleen population (Fig. 5.1 first row). However, in the negatively selected populations, the percentage of Lyt 2^+ and L3T4$^+$ cells increased about 3-fold with very few (1.4% L3T4$^+$ and 2.7% Lyt 2^+) cells of the depleted phenotype appearing after 3 days of culture (Fig.5.1 second and third rows).

The requirements for stimulating the proliferation of the T cell subsets was measured using the MTT (Table 5.3 A-D) and [^{3}H]-thymidine uptake assays (Table 5.3 E-H). The results obtained from these assays are similar, although the two methods measure slightly different parameters: MTT measures surviving cells, while thymidine measures proliferation.Thus, as also shown in the previous chapter, PMA and Cal in combination are sufficient to stimulate unseparated spleen cells from both naive and memory mice (Table 5.3 A,B and E,F, second column). The L3T4$^+$ cells can be stimulated directly by the drugs to proliferate (Table 5.3 D & H), however the ability of Lyt 2^+ cells to respond was much lower (Table 5.3 C & G, second column). Addition of 5 U/ml human recombinant IL-2 increased the proliferative capacity of cells in the PMA and Cal treated unseparated (Table 5.3 A,B and E,F third col.) and L3T4$^+$ cultures to some extent (1.14 and 1.03-fold, respectively Table 5.3 D,H). In contrast, the proliferative capacity of the Lyt 2^+ cells increased 2.3-fold when the culture was supplemented with exogenous IL-2. (Table 5.3 C & G). The same amount of IL-2 alone resulted in only a minor increase in proliferation for each population (Table 5.3 1st col. vs.4th col.).

The lytic capacity of these subsets has also been measured (Table 5.4). Neither of the subsets can be stimulated with PMA and Cal to kill MHC-restricted target cells (Table 5.4 C,D) but, if exogeneous IL-2 is provided, the Lyt 2^+ cells are able to mediate specific lysis (Table 5.4 C, 3d col.). The results suggest that stimulation of the lytic activity of Lyt 2^+ cells by PMA and Cal is totally dependent on the presence of IL-2.

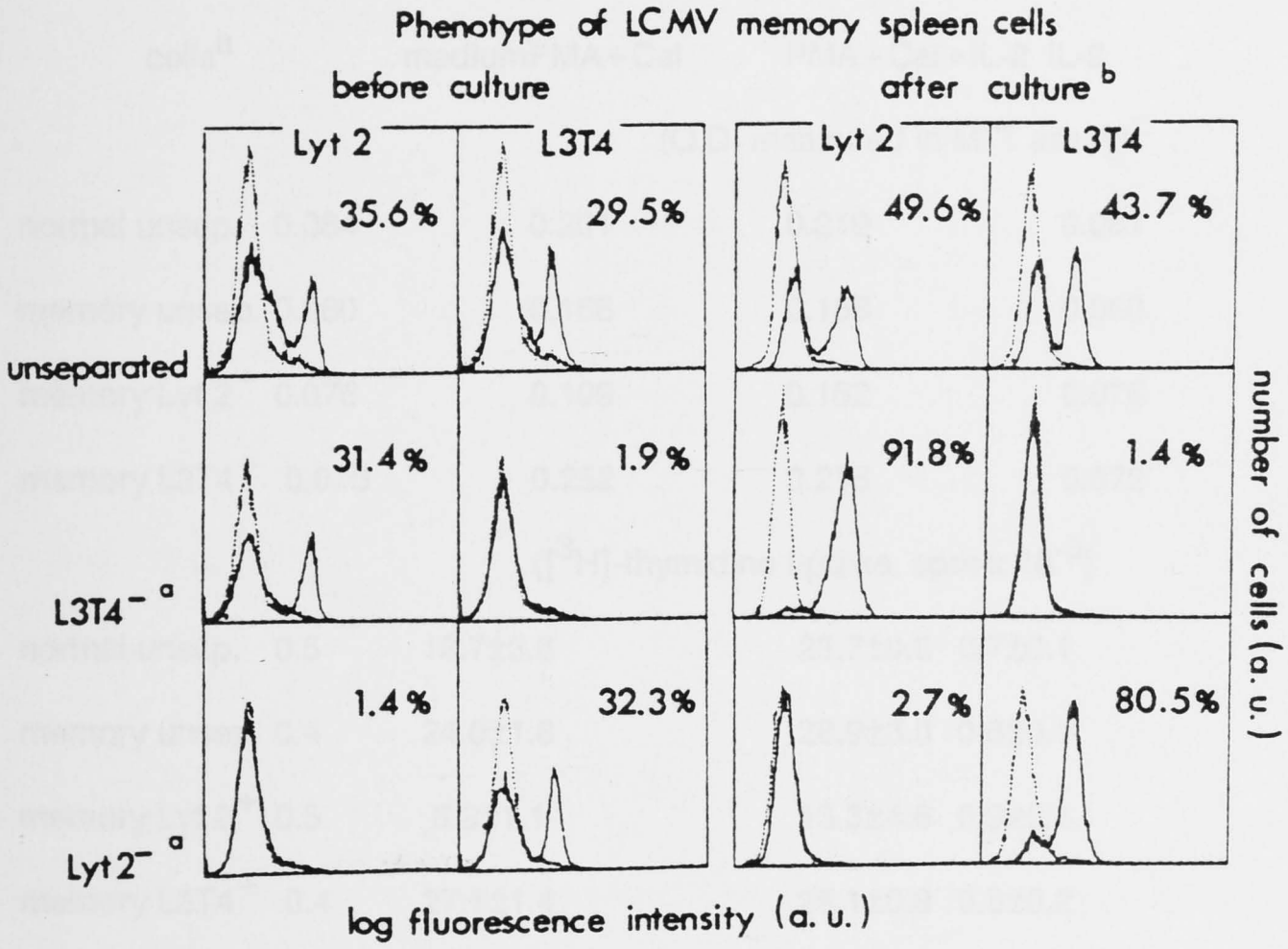


Fig. 5.1 Phenotypic analysis of negatively selected T cell subsets before and after stimulation with PMA+Cal+IL-2.

a) CBAxB6)F_1 spleen cells, 6 weeks after priming with LCMV

b) spleen cells were depleted by monoclonal antibody (RL 172, 3.168.8) and complement treatment.

c) cells were cultured for 3 days in the presence of 1 ng/ml PMA, 100 ng/ml Cal and 5 U /ml IL-2. Aliquots of viable cells obtained on ficoll-hypaque were stained with biotinylated antibodies and avidin-FITC. Dotted lines represent the binding of avidin-FITC alone. Percentage values represent the proportion of positive cells.

Table 5.3 Proliferation of T cell subsets upon PMA+Cal stimulation.

Group	cells[a]		Proliferation after culture[b] with medium	PMA+Cal	PMA+Cal+IL-2	IL-2
			(O.D. measured in MTT assay)[c]			
A	normal unsep.	0.084	0.201		0.219	0.081
B	memory unsep.	0.060	0.168		0.158	0.060
C	memory Lyt 2^+	0.076	0.109		0.152	0.076
D	memory L3T4$^+$	0.076	0.252		0.218	0.072
			([^{3}H]-thymidine uptake, cpm x 10^{-3})			
E	normal unsep.	0.5	18.7±3.6		23.7±0.6	0.7±0.1
F	memory unsep.	0.4	24.0±1.8		28.9±3.0	0.6±0.1
G	memory Lyt 2^+	0.5	6.9±1.1		15.3±4.6	0.9±0.1
H	memory L3T4$^+$	0.4	27.1±1.4		28.1±0.8	0.8±0.2

a) Spleen cells and depletion are the same as in Fig. 5.1

b) $5x10^4$ cells/well were incubated for 48 hr with 1 ng/ml PMA, 100 ng/ml Cal and/or with 5 U/ml IL-2.

c) Mean of triplicate samples is shown, S.E. < 0.048.

Table 5.4 The role of IL-2 in the stimulation of lytic activity of T cell subsets by PMA+Cal.

Group	cells[a]	% specific lysis after incubation[b]			
		medium	PMA+Cal	PMA+Cal+IL-2	IL-2
A	normal unsep.	ND.[c]	ND.	5.1	5.3
B	memory unsep.	6.8	50.0	51.9	28.8
C	memory Lyt 2^+	ND.	0	34.2	26.9
D	memory L3T4$^+$	ND.	0	3.7	0

a) spleen cells were prepared as in Fig.5.1.

b) 10^6 cells/well were cultured in 24 well trays for 3 days with 1 ng/ml PMA, 100 ng/ml Cal and/or 5 U/ml IL-2. The targets are LCMV-infected MC57G cells, E:T ratio = 30:1. Mean of triplicate samples is shown, S.E. < 5%.

c) not done

5.2.3 Expression of Ly 24 glycoprotein distinguishes memory and naive T cells.

As unprimed Ly 24^- lymphocytes become Ly 24^+ upon in vitro antigenic or mitogenic stimulation, the question arose as to whether in vivo primed lymphocytes can also be distinguished from unprimed cells by expression of this marker. Nylon wool enriched spleen cells from mice primed with LCMV 8-10 weeks previously were stained with biotinylated Ly 24 antibody followed by avidin-FITC, and the level of Ly 24 expression determined by single color FMF (Fig. 5.2 A). Cells were sorted by FMF to give about 20% Ly 24^+ and 50% Ly 24^- as described in Fig. 5.2. The level of contamination was checked by re-running the samples after sorting. The forward light scatter profile of Ly 24 positive and negative subsets is shown on Fig. 5.2 B. The Ly 24^+ cells tended to be slightly larger than the negative cells which could be due to the presence of more blast cells in this subset. Both populations were then cutured in the presence of PMA, Cal and IL-2 and their lytic activity checked against syngeneic, virus-infected target cells (Fig 5.3). The Ly 24^+ population gave rise to all the LCMV- specific cytotoxic activity and seemed to be enriched in lytic activity in comparison to the unsorted cells. The lytic activity of unsorted but stained cells did not differ greatly from that of the unstained sample, indicating that the antibody and the sorting procedure probably had neither an inhibitory nor a stimulatory effect on the cytotoxic activity of these cells.

The specificity of the killing was also studied using different target cells (Fig. 5.4). Ly 24^+ cells killed their specific target type and also showed lectin-dependent lytic capacity against phytohemagglutinin (PHA) treated target cells while Ly 24^- cells did not kill these targets. However, Ly 24^- cells killed Con A treated target cells after culture in the presence of Con A (Fig. 5.5) suggesting that this population contained T cells and has the capacity to be lytic under appropriate conditions.

The results support the idea that in vivo primed T cells become Ly 24^+ thus allowing selection of this functionally distinct subset through expression of this differentiation antigen.

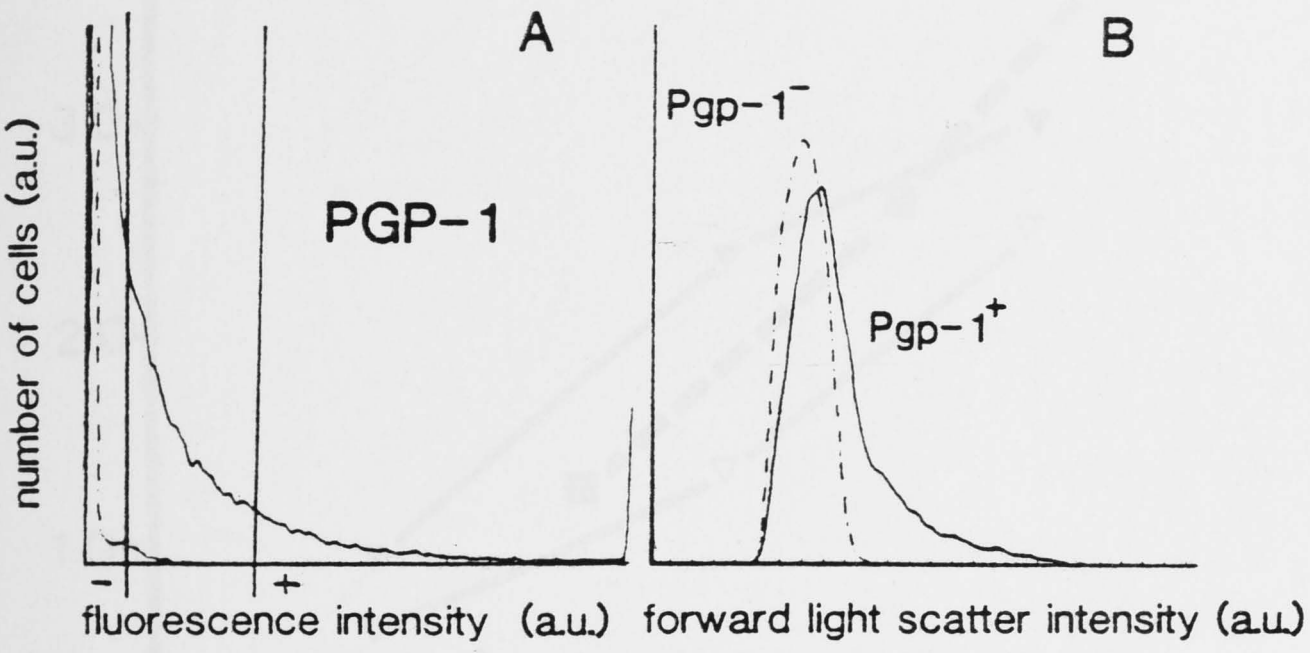


Fig. 5.2 Ly 24 (Pgp) expression on LCMV-immune spleen cells.

Spleen cells from (CBAxB6)F_1 mice were harvested 80 days after priming with LCMV. A: Aliquots of cells were stained with biotinylated anti-Ly 24 antibody followed by avidin – conjugated FITC. Of the nylon wool separated spleen cells 45.1% were Ly 24^+. Cells between channels 1 and 20 were sorted into Ly 24^- (56.6%) and between channels 60 and 255 into Ly 24^+ (20%) subpopulations.

B: The forward light scatter analysis of the sorted T cell subsets.

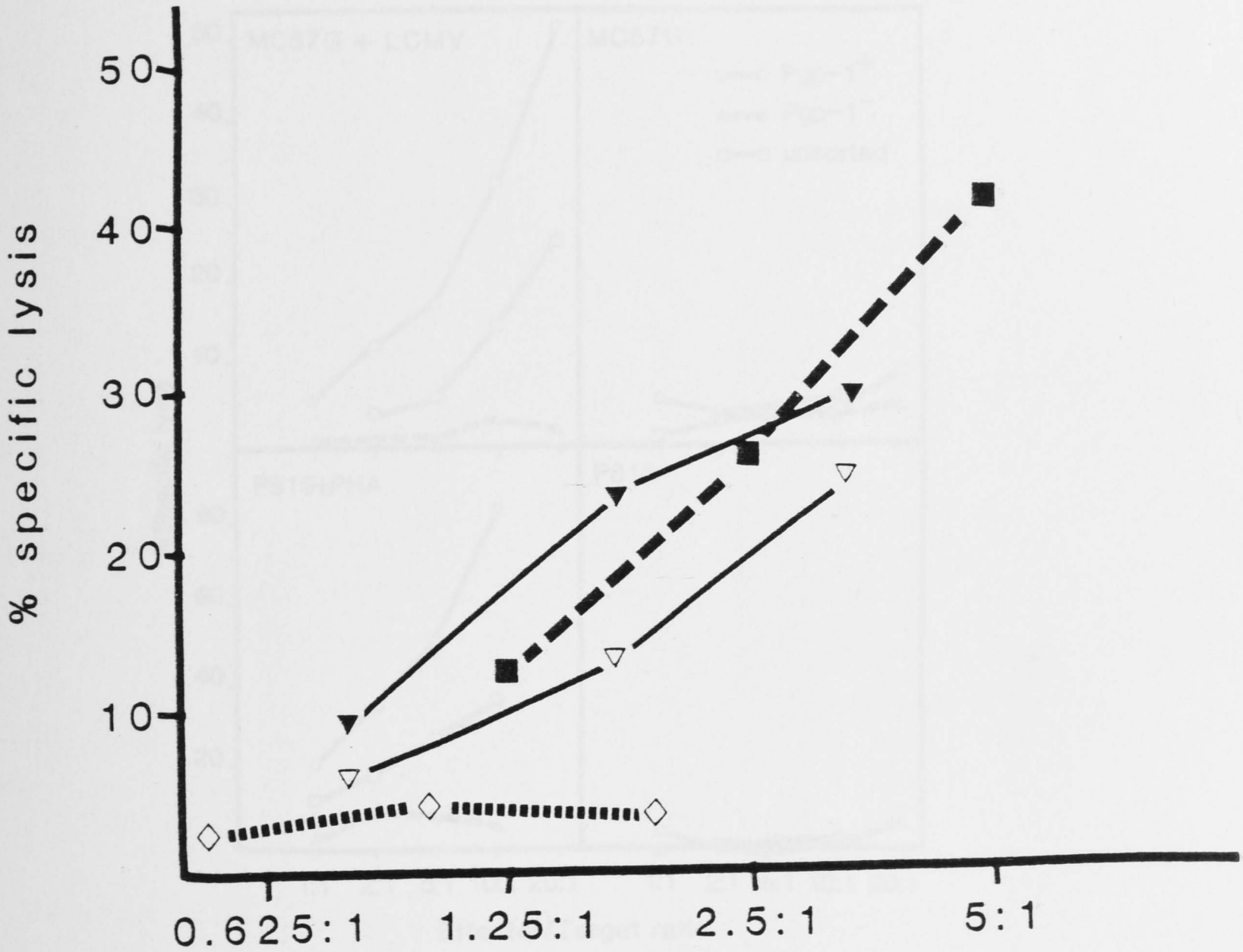


Fig. 5.3 Functional differencies between Ly 24^+ and Ly 24^- populations of immune T cells.

Spleen cells from (CBAxB6)F_1 mice primed with LCMV 10 weeks before the assay were sorted into Ly 24^+ (■) and Ly 24^- (◇) populations. The sorted cells, the stained, unsorted (▼) and the original unstained population (▽) were cultured at 10^4 cells/well for the Ly 24^+ cells and $3x10^4$ cells/well for the other groups. Cultures contained 1 ng/ml PMA, 100 ng/ml CaI and 10 U/ml IL-2. CTL activity against LCMV-infected MC57G cells was measured 6 days later. The average of duplicate samples is shown, S.E. < 5%.

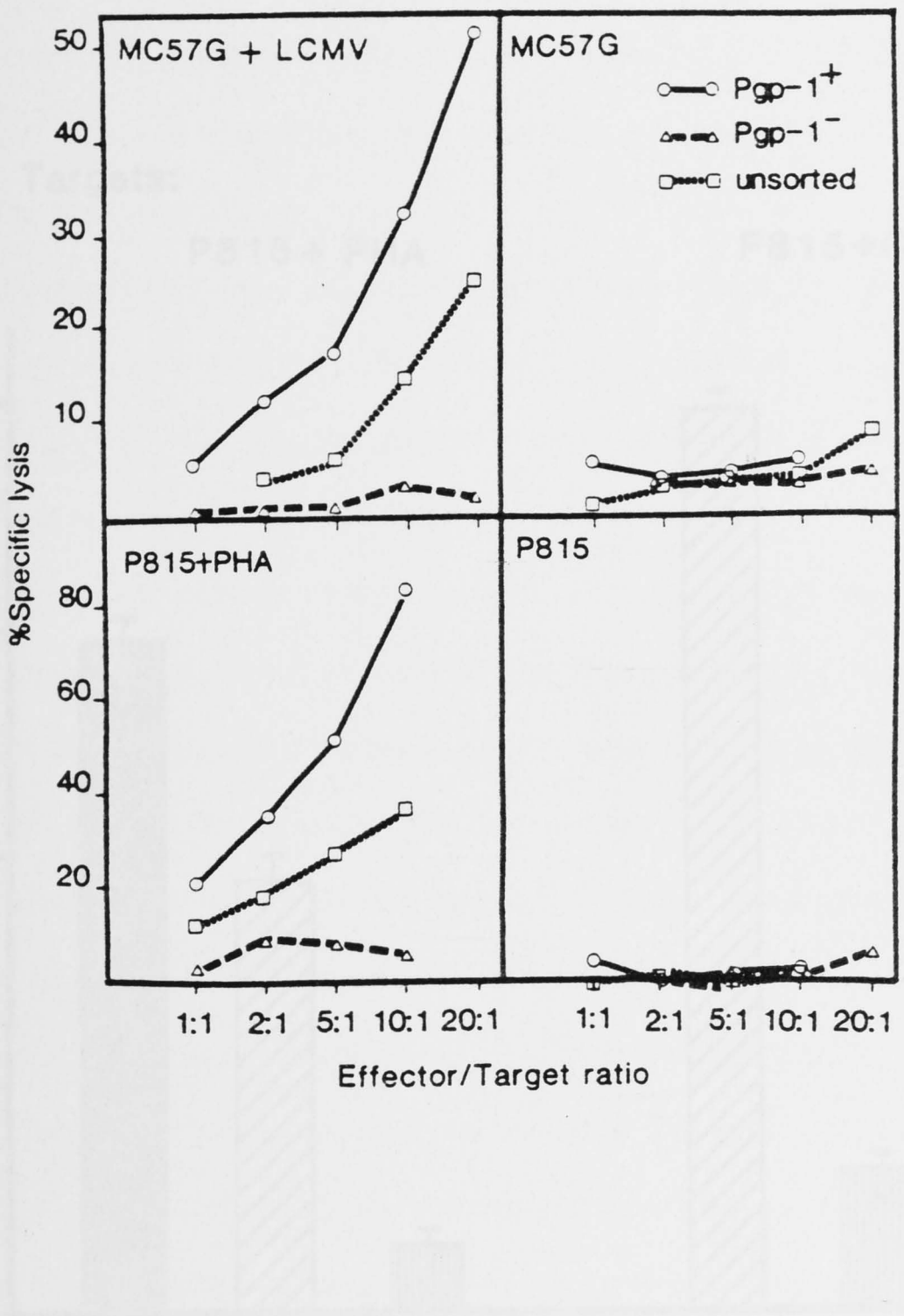


Fig.5.4 Specificity of killing by Ly 24$^+$ cells stimulated with PMA and Cal.

Ly 24$^+$, Ly 24$^-$ and stained, unsorted spleen cells obtained by FMF from (CBAxB6)F_1 mice primed with LCMV 12 weeks before the assay, were cultured for 4 days at 1.5 x 10^5 cells/well. Cells from each group were pooled and their cytotoxic activity measured on different target cells (S.E. of mean of triplicate wells <3.3%).

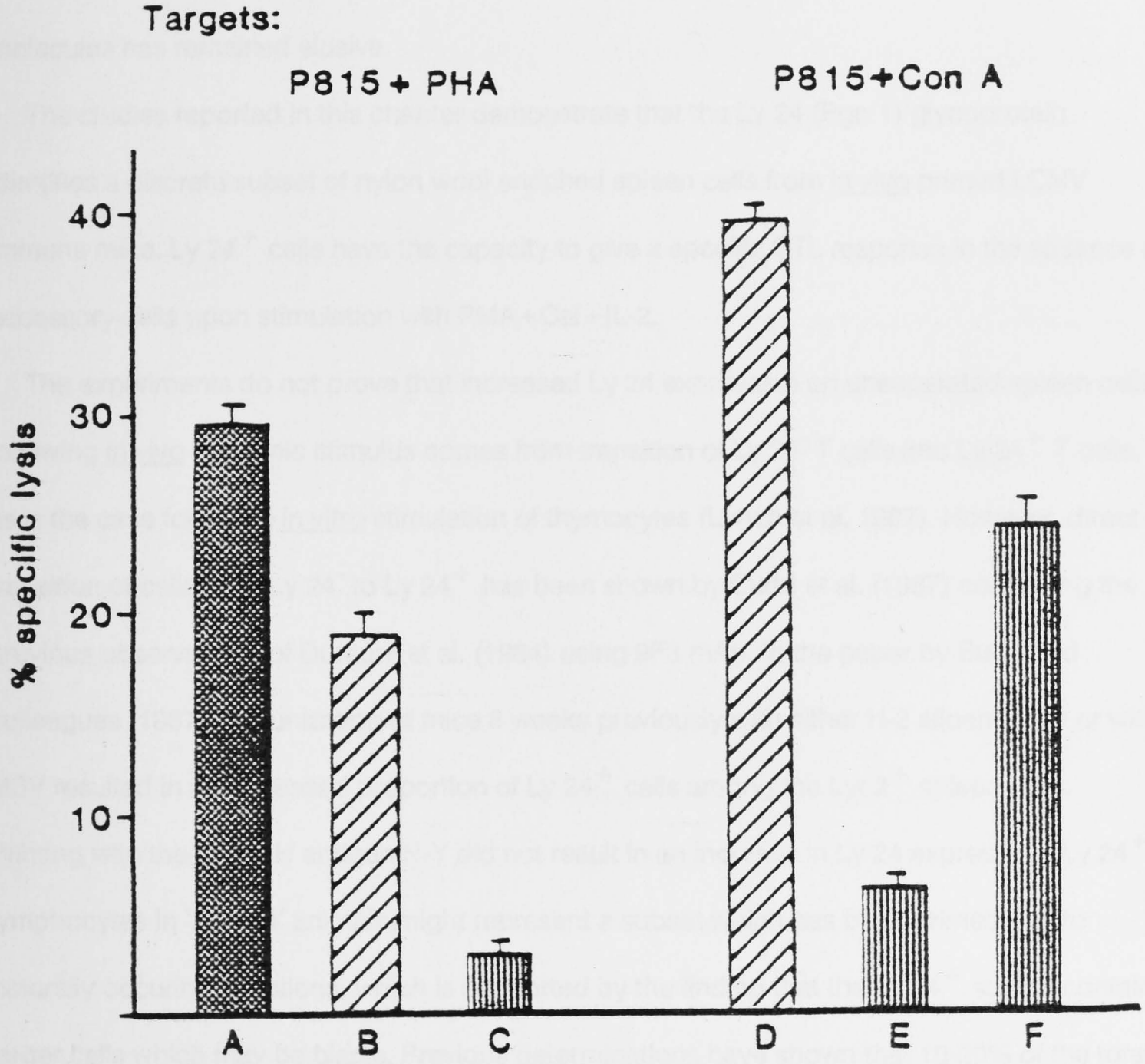


Fig. 5.5 Lectin-dependent killing by the sorted T cells.

Spleen cells from B6 unprimed mice were sorted into Ly 24^+ (▨) and Ly 24^- (▥) populations (unsorted cells: ▨) and cultured for 7 days at 2×10^4 cells/well in the presence of PMA and Cal as usual (A,B,C,D,E) or in the presence of 0.5 μg/ml Con A (F). The lytic activity was tested against PHA-treated (A-B) or Con A treated (D-F) P815 target cells. E:T ratio is 2.5:1, mean of triplicate samples is shown.

5.3 Discussion

Despite their unique properties, such as increased frequency and the presence of high avidity antigen receptors, identification and separation of memory T cells by cell surface molecules has remained elusive.

The studies reported in this chapter demonstrate that the Ly 24 (Pgp 1) glycoprotein identifies a discrete subset of nylon wool enriched spleen cells from in vivo primed LCMV immune mice. Ly 24^+ cells have the capacity to give a specific CTL response in the absence of accessory cells upon stimulation with PMA + Cal + IL-2.

The experiments do not prove that increased Ly 24 expression on unseparated spleen cells following in vivo antigenic stimulus comes from transition of Ly 24^- T cells into Ly 24^+ T cells, as is the case following in vitro stimulation of thymocytes (Lynch et al. 1987). However, direct transition of cells from Ly 24^- to Ly 24^+ has been shown by Budd et al. (1987) confirming the previous observations of Dumont et al. (1984) using 9F3 mAb. In the paper by Budd and colleagues (1987) immunization of mice 8 weeks previously with either H-2 alloantigens or with MSV resulted in an increased proportion of Ly 24^+ cells among the Lyt 2^+ spleen cells. Priming with the minor H antigen H-Y did not result in an increase in Ly 24 expression. Ly 24^+ lymphocytes in 'normal' animals might represent a subset which has been primed due to naturally occuring infections, which is supported by the finding that the Ly 24^+ subset contains larger cells which may be blasts. Previous determinations have shown that 10-20% of the total pool of splenic T lymphocytes in vivo are naturally activated cells. They tend to be larger and can be selectively grown in vitro by IL-2 (Pereira et al. 1985). Similarly, Lynch has found that proliferation of Ly 24^+ but not Ly 24^- cells can be induced by IL-2 in vitro (manuscript in prep.).

The functional properties of the Ly 24^- cells have also been studied: they did not kill virus infected syngeneic, allogeneic or lectin treated targets upon PMA stimulation, but lysed Con A-treated targets when they were stimulated in the presence of Con A. Thus the sorted Ly 24^- cells did contain T cells. The role of Con A in mediating LDCC has been the subject of several investigations (Gately and Martz, 1977; Berke et al. 1981a & b; Bonavida and Katz, 1985). It has been shown that LDCC and cell-mediated cytotoxicity (CMC) follow the same pathway of lysis, but the exact mechanism is not clear. The possibilities are that the lectin serves as a 'glue' to

bring effector and target cells in close proximity; that it activates T cells (Bonavida et al. 1977; Green et al 1978); or that it modifies MHC class I molecules (Berke et al 1981a & b) or other structures on the targets (Bonavida and Katz, 1985). What is known is that virtually all mature CTL have the ability to mediate Con A-dependent killing.

There are conflicting reports in the literature about the ability of tumor promoters and calcium ionophores to activate the helper and cytotoxic T cell subsets. It has been reported recently that these drugs stimulate cloned T helper cells to proliferate and to produce lymphokine, but do not activate cytotoxic T cell clones to mediate cytotoxic effector function (Albert et al. 1985; Isakov and Altman, 1986). Truneh et al. (1985) obtained similar results with alloreactive T cell subsets enriched for $Lyt2^+$ or $L3T4^+$ cells, respectively. In contrast, Berebbi et al. (1987) found that the drugs increased the ability of cloned CTL to form conjugates and mediate lysis not only of targets to which they were sensitised but also irrelevant targets. In addition they found similarities between PMA and CaI activation of lymphokine secretion from cloned helper cells and triggering of lysis by CTL. The discrepancies between these reports may be explained by differencies in the concentration range of drugs used for stimulation and by differencies among CTL clones. It has also been suggested that the outcome of stimulation depends on the stage of the CTL cell cycle at the time of treatment (Ashwell et al. 1986).

Antigenic stimulation and exposure to mitogens or PMA have all been shown to cause rapid downregulation of the T3/TCR complex on a T cell hybridoma, T cell clones, thymic and peripheral T cells and T cell tumors (Ando et al, 1985; Cantrell et al. 1985; Weyand et al. 1987). This can be accompanied by parallel downregulation of the CD4 molecule (Weyand et al. 1987; Hoxie et al. 1986; Solbach, 1982). Similar consequencies following PMA treatment were observed on nylon wool enriched spleen cells (Table 5.2), but two colour FMF would be necessary to determine the exact phenotypic changes within the T cell subsets. The significance of the phosphorylation and downregulation process is not clearly understood, but it can be thought to reflect a negative-feedback mechanism that functions to reduce the surface expression of T3/TCR complex after antigen recognition, thereby terminating further activation by antigen. Parallel downregulation of the CD4 molecules suggests that they may form a physical association with TCR (Fazekas de St.Groth et al. 1986).

Budd et al. (1987a and b) have found that Ly 24 is expressed on both Lyt 2^+ and L3T4$^+$ peripheral T cells, with a slightly higher proportion of the former subset being positive. From studying these subsets it seems that, although the positively selected Ly 24^+ population contains all the factors necessary to give rise to LCMV- specific secondary CTL activity upon PMA stimulation (Figs.5.4 & 5.5), the activation requirements are different for Lyt 2^+ and L3T4$^+$ cells. Thus, incubation with PMA and Cal does not induce CTL function of Lyt 2^+ cells in the absence of IL-2 or IL-2 producing L3T4$^+$ cells, though the lymphocytes may still proliferate. Whether proliferation of such Lyt 2^+ L3T4$^-$ lymphocyte populations reflects endogeneous IL-2 production by Lyt 2 cells (Widmer and Bach, 1981; Kelso and Glasebrook, 1984) has not been established. A logical interpretation of the experiments would be that any IL-2 originating from the Lyt 2^+ subset is consumed to maintain cell division, and is insufficient to induce CTL function. Alternatively, cells other than the Lyt 2^+ population may be proliferating, or lymphocyte division may be maintained by a different pathway (Koretzky et al. 1983). Recent findings suggest that IL-4 plays an important role in stimulating some T cell subsets to proliferation. (Mossmann et al. 1986).

The observation that there are differences between Lyt 2^+ and L3T4$^+$ T cells in the conditions necessary for stimulation indicate that there are differences in the signal requirements for activation of lymphokine production and cytotoxicity. L3T4$^+$ cells can be directly activated by PMA and Cal, however stimulation of Lyt 2^+ cells is secondary to the release of IL-2 produced by L3T4$^+$ cells. The results also suggest that cytolytic function does not necessarily represent an end-stage of maturation (Mitchison and Pettersson, 1983; Bandeira et al. 1987). Alternatively, effector CTL may revert into non-cytolytic memory cells observed for cloned CTL (Orosz et al. 1985).

Chapter 6

Cytotoxic T cell response to recombinant vaccinia virus expressing the H-2K^d alloantigen.

6.1 Introduction

The available data on MHC-restricted and alloimmune T cell-responses suggest that virus-immune and alloreactive T cell precursors occur at similar frequencies in virus primed and unprimed mice, respectively. The high frequency of T lymphocytes recognizing allelic variants of MHC products may be explained by a high degree of similarity between complexes composed of syngeneic MHC-products + peptide and foreign MHC gene products (Burakoff et al. 1978; von Boehmer et al. 1979; Sredni and Schwartz, 1980; Townsend et al. 1985; Maryanski et al.1986; Bjorkman et al. 1987 a and b). Another explanation resulting from Jerne's hypothesis (1971) is that there are germ line-encoded receptors for both self and allogeneic MHC specificities. It has been suggested (von Boehmer et al. 1978) that high affinity self-reactive clones are eliminated during ontogeny, while allo-reactive clones remain at a high level.

The results discussed in Chapter 4 and 5 suggest that alloreactivity does not result from previous encounters with environmental infectious agents. Furthermore, the lack of stimulation of alloreactivity from naive spleen cells by PMA and CaI indicates that alloreactive clones do not exist as activated cells in these mice. However, in contrast to primary MHC-restricted anti-viral T cell-responses, alloreactive CTLp can be activated at high frequencies in vitro.

Thus, the conventional method for testing alloreactivity in mixed lymphocyte cultures does not distinguish between primary responses induced in vitro and secondary alloreactivity due to in vivo priming. Stimulation of memory cells in vitro in the absence of antigen with PMA and CaI provides a protocol for analysing the pCTL-frequency resulting from only the antigen used for in vivo priming. In this chapter, use is made of a recombinant vaccinia virus which contains the coding sequence for the H-2K^d allo-antigen. Vaccinia can be engineered to express cloned genes encoding immunologically important proteins from a variety of unrelated viruses (Smith et al. 1983; Panicali et al. 1983; Bennink et al. 1984; Wiktor et al. 1984; Mackett et al. 1985; Andrew et al. 1987), tumor specific antigens (Lothe et al. 1987) and histocompatibility antigens (Coupar et al. 1986). The vaccinia H-2K^d recombinant virus has been shown in previous experiments to generate an H-2K^d-specific primary cytotoxic T cell response when injected into H-2 different

mice and to act as a target for alloreactive and H-2K^{d}-restricted vaccinia virus-specific cytotoxic T cells when used to infect allogeneic (H-2^{k}) L929 cells (Coupar et al. 1986).

The present experiments are concerned with determining whether there is a T cell repertoire for the recognition of H-2K^{d} + vaccinia virus that is separate from that for H-2K^{d} alone in H-2^{b} mice. This has been tested at the clonal level using limiting dilution analysis, and has also been analyzed in H-2^{b} mice made tolerant of H-2K^{d} by injection of H-2^{dxb} (F_1) cells as neonates.

6.2 Results

6.2.1 Primary and secondary CTL responses against the recombinant virus.

Priming B6 (H-2^b) mice with the control vaccinia virus (V-TK$^-$) led to a potent anti-viral CTL response with comparable lysis of MC57G (H-2^b) targets infected with V-K^d and V-TK$^-$ (line 1, Table 6.1). There was also some lysis of the KD2SV (H-2^d) vaccinia-infected target, but this was much less than that caused by syngeneic, virus-primed Balb/c (H-2^d) effectors (compare lines 1 and 3, Table 6.1), and may represent non-specific cytotoxicity. Infection of B6 mice with V-K^d did not, however, lead to higher levels of cytotoxicity for virus-infected (as compared with uninfected) targets (line 2, Table 6.1). Thus, for primary <u>in vivo</u> CTL tested in a bulk assay, exposure of H-2^b mice to vaccinia virus carrying the H-2K^d alloantigen does not allow the discrimination of a response to H-2K^d - vaccinia virus from that to H-2K^d alone.

The secondary CTL response was measured 7 weeks later (Table 6.2) following restimulation with PMA and CaI <u>in vitro</u> by the method described in Chapter 4. As found for primary CTL, effectors primed with the recombinant virus had potent alloreactivity against H-2^d targets and also against virus-infected H-2^b targets (Table 6.2, 1st row). Third party H-2^k target cells infected with vaccinia-TK$^-$were not lysed. Very low levels of lysis (5-8%) were found when vacc-K^d was used to infect the cells (Table 6.2, columns 5 and 6).

6.2.2 Clonal distribution of the secondary response.

The precursor CTL frequencies against virus or alloantigen were determined for mice primed with the V-K^d virus construct following specific restimulation <u>in vitro</u> with antigen (Table 6.3) or by stimulation with PMA + CaI + IL-2 (Table 6.4). The latter method has been described in greater detail in the LCMV system (Chapter 4).

The frequency of alloreactive CTLp in H-2^b mice primed with V-K^d and restimulated with allogeneic cells is 1/438 compared with 1/451 for V-K^d primed cells restimulated with Virus-infected H-2$K^k$$D^d$ cells and

Table 6.1 Primary CTL response against recombinant vaccinia virus.

Mice[a]	Priming vaccinia[b]		% specific lysis[c] H-2^b			H-2^d		
	TK$^-$	+K^d	V-K^d	V-TK$^-$	U	V-K^d	V-TK$^-$	U
B6	+		36.1	32.6	10.7	15.8	14.1	3.8
B6		+	46.1	46.6	23.0	50.2	31.7	42.3
Balb/c	+		21.9	11.9	4.7	61.4	43.9	11.1

a) 2 mice/group, 6 days after priming

b) 10^7 PFU virus i.v.

c) 100:1 E:T ratio, results are calculated by linear regression of data at different E:T ratios. Mean of triplicate samples is shown, S.E. < 5%. The targets are MC57G (H-2^b), and KD2SV (H-2^d) cells, infected with the recombinant virus (V-K^d), the control virus (V-TK$^-$) or uninfected (U); spontaneous lysis less than 27%.

Table 6.2 Secondary CTL response following stimulation of recombinant virus primed spleen cells with PMA and Cal[a].

	% specific lysis[c]							
	H-2^b		H-2^d		H-2^k		H-2^{kd}	
Priming[b]	V-TK$^-$	U	V-TK$^-$	U	V-TK$^-$	V+K^d		U
vacc-K^d	20	0	36	38	0	8		0
vacc TK$^-$	29	0	7	8	0	3		0
H-2^d cells	1	1	46	44	0	5		20
Nil	1	0	5	6	0	1		0

a) 5 days stimulation in the presence of 1 ng/ml PMA 100 ng/ml Cal and 5 U/ml IL-2.

b) B6 mice were immunized with 10^7 PFU virus or 2×10^7 P815 cells i.v. 51 and 37 days before assay, respectively.

c) MC57G (H-2^b), KD2SV (H-2^d), L929 (H-2^k) and YAC (H-2^a) target cells were used at 60:1 E:T ratio. Mean of triplicate samples is shown, S.E. < 5%. Abbreviations as in Table 6.1.

Table 6.3 LDA of pCTL frequencies after antigen-specific restimulation in vitro.

Group	Primed with:[a]	In vitro stimulation[b]	Reciprocal frequencies[c]			
			H-2^d			H-2^b
			V-TK$^-$	U	V-K^d	V-TK$^-$
A	V-K^d	H-2K^{k}D^d	-	438	-	-
B	V-K^d	H-2K^{k}D^d + vacc	295		451	-
C	V-K^d	H-2^b + vacc	-	-	-	643
D	V-TK$^-$	H-2K^{k}D^d	-	230	-	-
E	V-TK$^-$	H-2^b + vacc	-	-	-	360
F	P815	H-2K^{k}D^d	-	512	-	-
G	P815	H-2K^{k}D^d + vacc	-	-	923	-
H	nil	H-2K^{k}D^d	-	318	-	-
I	nil	H-2K^{k}D^d + vacc	-	-	1190	-
J	V-TK$^-$	H-2K^{k}D^d + vacc	937		-	-
K	V-TK$^-$	H-2^b + vacc	-	-	-	995

a) B6 (A-I) or (Balb/c X B6)F_1 mice were primed with 10^7 PFU virus or 2×10^7 cells i.v., for 12 or 10 weeks, respectively.

b) 10^5 gamma-irradiated stimulators/well, 8 day culture

c) Calculated by linear regression, $r > 0.9$.

Targets: KD2SV and MC57G cells, infected as in Table 6.1.

Table 6.4 LDA of pCTL frequencies after stimulation with PMA and Cal[a].

Gp	Priming with:[b]	Reciprocal frequencies[c] H-2^d		H-2^b		H-2^k
		V-TK$^-$	U	V-TK$^-$	U	U
A	V-K^d	394	882	1384	N.D.	>18000
B	V-TK$^-$	>6000	>6000	540	>20000	N.D.
C	P815	298*	322	N.D.	N.D.	>180000
D	Nil	>7000	>7000	>10000	N.D.	>250000

a) 8 days incubation with 1 ng/ml PMA, 100 ng/ml Cal and saturating amounts of IL-2 (see Chapters 2 & 4).

b) as in Table 6.3

c) Calculated as described in Table 6.3, r>0.92, except * 0.864, and (>) where linear regression analysis cannot be applied. Targets are as in Table 6.1.

tested on V-K^d infected H-2^b targets (Groups A and B, Table 6.3). The frequency of self H-2^b MHC-restricted T cells in these mice was slightly lower (1.643, group C, Table 6.3). V-TK^- - infected KD2SV target cells were lysed at a slightly higher level, when spleen cells from V-K^d primed H-2^b mice were restimulated with H-2^d cells infected with vaccinia virus, than H-2^b cells infected with V-K^d (1/295 compared with 1/451). The frequency of self MHC-restricted anti-viral CTLp (1/643) is slightly lower than that from mice infected with vaccinia virus (1/360) while that of CTLp specific for uninfected H-2^d targets (1/438) is of similar magnitude to that of mice injected with P815 (H-2^b) cells (1/512), or from 'naive' mice stimulated in vitro with H-2$K^d D^k$ cells (1/318). Stimulation of the different effectors with vaccinia TK^- virus infected H-$2K^d$ antigen presenting cells resulted in about 1/1000 pCTL frequency when the H-$2K^d$ served as alloantigen on the surface of H-2^b targets (Table 6.3, G and I) but the frequency was more than two-fold higher (1/451) when the H-$2K^d$ might have served as a restriction element as well (Table 6.3, B).

The results in Table 6.4 show the pCTL frequencies due to in vivo priming either with virus (B) or alloantigen (C) or both, using the virus construct (A) and after restimulation with PMA and Cal. As mentioned previously, the main difference between the two approaches is that the conventional LDA uses allogeneic stimulator cells. Thus, while it measures mainly in vitro induced primary allo-immune effector-T cells there may also be some secondary lytic activity from effectors generated in vitro from in vivo primed CTL-precursors. Therefore, the results from antigen-independent stimulation, summarized in Table 6.4, could be considered to reflect the consequencies of priming with the recombinant virus more precisely.

Priming B6 mice with V-K^d led to a higher frequency of CTLp for virus-infected, as compared with uninfected, H-2^d targets (2.2-fold, line 1, Table 6.4). This difference did not mean that the virus-infected targets were more susceptible to lysis by alloreactive T cells, as the frequency of CTLp assayed on uninfected targets was almost the same from mice primed with P815 cells (line 3, Table 6.4). It thus seemed possible that there could be at least some T cells reactive to V-K^d, rather than to K^d alone, in the H-2^b mice.

The V-TK^- virus was more potent at priming H-2^b - restricted CTLp than was V-K^d (1/540, compared with 1/1384, Table 6.2). This was also apparent following restimulation with virus-infected cells (Groups C and E, Table 6.3). This could simply reflect that V-TK^- grows better and is

more immunogenic, or that the presence of K^d in some way skews the response away from H-2^b + vaccinia virus.

The assumption that some CTL clones of V-K^d primed B6 mice are specific to vaccinia in the context of H-$2K^d$ was checked by a well-split analysis at the clonal level (Fig. 6.1). Gradually increasing number of cells (200-400-800) were cultured in the presence of PMA and CaI as described previously (Chapter 4) and the cytotoxic activity of each well was tested against two targets. This approach gives more information about the CTL clones than LDA. At 200 and 400 cells/well ~~where the probability of clonality is high, according to the results in Table 6.4~~, some clones from the recombinant virus primed mice showed minimal evidence of cytotoxicity for vaccinia-infected H-2^d targets but not for uninfected cells (panel A, Fig. 6.1). The level of specific lysis is very low (slightly above the 5% limit), thus to show definitively that these cells are specific for H-$2K^d$ + virus it would be necessary to isolate the clones and characterise their fine-specificity. In any case, these few, minimally potent CTLp cannot be considered to reflect the presence of a significant repetoire for K^d + vaccina virus in H-2^b mice.

The well-split analysis can also distinguish between MHC-restricted and allo-immune clones. Effectors from vaccinia TK^- virus primed mice were found to kill only virus-infected H-2^b targets when analysed at 200 and 400 cells per well (panel C, Fig 6.1). When the clones of V-K^d primed mice were split onto virus-infected H-2^b and H-2^d targets (panel B, Fig. 6.1), at 200 cells/well all the positive wells were functionally 'single positives', at 400 cells/well only 1 well seemed to show double specificity (11%), whereas at 800 cells/well lysis in 19% of the positive wells was MHC-restricted, 25% exhibited anti-allo killing, and the remaining wells had double specificity. The probability of clonality at this cell number is less than 1 (i.e. individual wells may contain more than 1 CTL precursor), the assay suggests that different clones are responsible for the MHC-restricted and for the allo-immune CTL responses, respectively.

6.2.3 Secondary response of mice tolerant to H-2^d

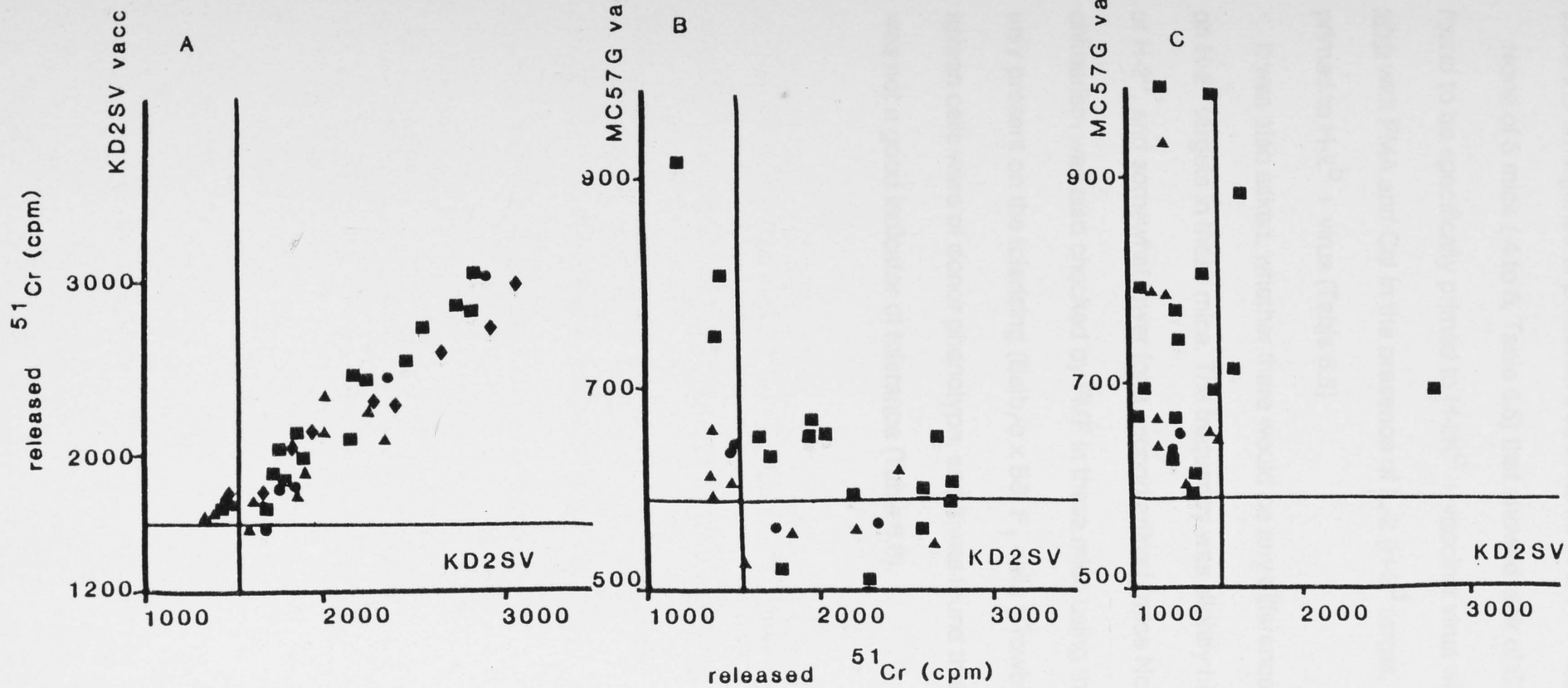


Fig.6.1 Specificity of pCTL at clonal level

Effector spleen cells were cultured for 8 days in the presence of 1 ng/ml PMA, 100 ng/ml Cal and saturating amounts of IL-2. Wells were split in two and lytic activity of cells measured against the targets shown on the figure. The spleen cells tested in A and B were primed with V-Kd, while those tested in C were from mice given V-Th⁻. The cultures were established with 200 (▲), 400 (●) or 800 (■) cells per well.

H-2^b mice were tolerized to H-$2K^d$ by immunization with (H-2^{bxd}) F_1 spleen cells 24 hr within birth. It was asked whether abrogation of the alloreactive response for H-$2K^d$ in H-2^b mice would allow the emergence of precursors specific for H-$2K^d$ + vaccinia virus.

None of 3 mice (4 to 6, Table 6.5) that showed lack of CTL effector function for H-2^d were found to be specifically primed to H-$2K^d$ + vaccinia virus when spleen cells were expanded in vitro with PMA and CaI in the presence of IL-2 (H-2^d target, Table 6.5). All three however, were primed to H-2^b + virus (Table 6.5)

It was also asked, whether there would be any differences for CTLp recognizing V-K^d or V-TK^- on H-2^b targets in these mice. The frequency was slightly higher for one mouse that was tolerant of H-2^d, and somewhat lower for a second animal (mice No 13 and 15, Table 6,6). The level of chimerism was also checked by FMF in these mice using the Ly 24.2 marker, which should be only present on the tolerising (Balb/c x B6) F_1 cells (Trowbridge et al. 1982). Less than 10% of spleen cells were of donor phenotype, and it was found that the number of these cells present was not a good indicator of tolerance (Table 6.6).

Table 6.6 Analysis of pCTL frequencies to recombinant vaccinia virus in H-2^d-tolerant mice.

Mouse No.	Tol. ind.[a]	% Ly 24.1$^+$ spl	% Ly 24.1$^+$ Thy	% specific[b] lysis (H-2^d)	reciprocal pCTL frequencies[c] d	b+V-TK$^-$	b+VK^d
1-3	-	0	0	74.0	1002	735	380
4	+	9.3	0	1.4	25600*	469	306
5	+	7.4	0.2	29.5	1420	192	145
6	+	2.1	0	5.1	1451$^\#$	169	451

a) Tolerance induction with2 x 10^7 (Balb/c x B6)F_1 spleen cells i.v. within 24h of birth.

b) Effectors primed in MLR with gamma-irradiated Balb/c spleen cells. Effector cells (3200/well) were cultured for 8 days. Mean of 16 wells/group is shown, S.E. < 5%.

c) Calculated as described (Chapter 2). r> 0.9, except for * (0.849) and $^\#$ 0.884. Targets are KD2SV and MC57G, abbreviations as in Table 6.1.

Table 6.5 Secondary CTL activity of H-2^d tolerant mice against the recombinant vaccinia virus.

Mouse No.	Tol. ind.[a]	% specific lysis(H-2^d)[b]	% specific lysis[c] H-2^d +V-K^d A	H-2^d +V-TK$^-$ B	H-2^d U C	H-2^b +V-TK$^-$ D	H-2^b U E
1-3	-	15	20	17	17	28	2
4	+	0	3	4	3	36	6
5	+	2	0	2	1	40	3
6	+	0	0	0	0	29	3
7-9	-	ND	22	22	0	20	ND

a) 1-6 B6 mice, 7-9 (Balb/c x B6)F_1 mice. 4-6: tolerance induction with 2×10^7 (Balb/c x B6) F_1 spleen cells injected within 24 h of birth.

b) Effector cells were stimulated with gamma-irradiated Balb/c spleen cells for 4 days, E/T ratio is 15:1.

c) Effectors were stimulated with PMA and Cal for 4 days as described (Chapter 2). Targets as listed in Table 6.1. E:T ratio is 60:1.

6.3 Discussion

Vaccinia recombinants expressing foreign antigens are potentially useful as live vaccines against a variety of viruses and also in the preventing the spread or cure of tumors carrying tumor-specific antigens (rev. by Moss, 1985; Lathe et al. 1987); The results in this chapter provide information about the general characteristics of the CTL response induced by a recombinant vaccinia virus.

T cell memory induced by the virus construct containing H-2K^d was found to be quantitatively and qualitatively similar in the case of MHC-restricted anti-viral and allo-immune responses. One possibility is that infection of mice with the recombinant virus results in a common pathway of antigen-processing and presentation for the viral proteins and for alloantigens. The proteins may be denatured and fragmented in the Golgi-apparatus and returned to the cell surface together with class I molecules (Morrison et al. 1986; Germain, 1986; Townsend et al. 1985). The idea would then be that different H-2^b glycoproteins on the cell surface would present peptides derived from either the H-2K^d molecule or from vaccinia virus. The alternative model is that processing of virus proteins is mediated via the above mechanism, while the H-2K^d glycoprotein is fully expressed the surface of cells infected with V-K^d (von Boehmer et al. 1979; Braciale et al. 1981; Weiss et al. 1980). The fact that infection with V-K^d primes for a secondary response to the K^d molecule (as determined by stimulation with PMA + Cal + IL-2) expressed on H-2^d targets might be thought to favour this idea. Otherwise, it would be necessary to argue that the same peptide derived from H-K^d can associate with H-2^b (i.e. K^b or D^b) or H-2^d (i.e. K^d, D^d or L^d) glycoproteins, and that the T cell receptor can recognize this peptide regardless of the presenting MHC molecule.

Experiments performed at the clonal level indicated that there is little, if any, repertoire for H-2K^d + vaccinia virus in H-2^b mice. Further limiting dilution analysis in H-2^b mice that are tolerant to H-2^d would probably not have modified this conclusion, though this experiment would have been examined if sufficient time had been available. However the results are in general accord with the idea that MHC-restricted T cell recognition is generally selected towards low affinity for

self MHC in the thymus (Zinkernagel, 1978; Bevan and Fink, 1978; Doherty and Bennink, 1979) and that the odd cases of recognition of non-self MHC + virus represent an aberrant cross-reactivity (Doherty and Bennink, 1979). Selective proliferation of the small number of clones with a low level of cytotoxicity for H-2^d + vaccinia virus (Fig. 6.1) could have lead to such findings (Doherty and Bennink, 1979).

Others have looked for clones which are specific for both viral antigen and alloantigen. T cell clones with dual reactivity to both self + antigen and also alloantigen have been described by Kaye and Janeway (1984) and Kanagawa and Nagasawa (1987). In these studies it was found that monoclonal antibodies to the TCR of these clones inhibited antigenic and allogenic stimulation. However, Kanegawa and Nagasawa (1987) found different mAb were required to inhibit recognition of self+antigen in comparison to alloantigen suggesting that different epitopes on the TCR recognised these antigens.

The exact mechanism of neonatal tolerance induction and its maintenance is not known. It has been observed that unresponsiveness to alloantigen is associated with decreased helper and cytotoxic precursor T cell frequencies specific for the tolerogen (Nossal and Pike, 1981; Carnard et al. 1984). The experiments summarized in Table 6.5 support these observations, ~~since the LDA studies of neonatally tolerized mice showed one-hit kinetics. This is~~ and are in accord with the suggestion that the decrease in precursor frequency is due to the removal of T cells by clonal deletion (Nossal, 1983; Wood et al. 1984). In contrast, others have found that tolerized cells can be reactivated in vitro (Heeg and Wagner, 1985) and that suppression can be 'infectious' (Fitch et al. 1976; Gorczynski and MacRae, 1979; Vegh et al. 1980; Janossy et al. 1983). However, stimulation of the tolerized cells with PMA and CaI did not result in the appearance of clones reactive to the tolerogen, which seems to favour the clonal deletion theory, but it is not known what effect PMA and CaI stimulation would have on the putative active suppression.

The experiments also deal with the differencies between alloantigen-specific CTL activity in unprimed or in in vivo primed mice, by utilizing an in vitro restimulation protocol in the absence of added antigen. This procedure maybe of practical value for analysing transplantation systems. One application would be to follow cellular immunity in recipients after transplantation particularly where the number of cells available is low due to cytotoxic immunosuppressive agents.

Taken together,the experiments with recombinant vaccinia virus encoding the H-2K^d alloantigen, showed no obvious differences between MHC-restricted anti-viral and allo-immune CTL-responses. It is possible that there could be some specific suppression of the K^d + virus repertoire in H-2^b mice, but it would need to be argued that such suppressors can operate to overcome the effects of PMA + CaI + IL-2. It seems likely that T cells reactive to antigens other than foreign MHC glycoproteins are constrained by non-self antigens presented in the context of self MHC-glycoproteins encountered in the thymus.

Chapter 7

General discussion

The main aim of this thesis was to examine the characteristics of cytotoxic T cell activation during viral infection. These studies have demonstrated that virus-primed cytotoxic T lymphocytes (CTLp) undergo differentiation in vivo and develop into primary effector cells, and also into memory cells creating the basis of a secondary CTL response. The primed cells can be characterised by their requirements for activation which differ from those of naive cells and by surface expression for the Ly 24 antigen. This discussion will examine the requirements for T cell activation in primary and secondary T cell responses. The ways in which these studies should be extended will also be considered.

Cytotoxic T lymphocytes arise during the course of a cell-mediated immune response to viral infection. The properties of the primary anti-viral T cell response were studied following in vivo priming, as a potent primary CTL response to virus cannot be induced in vitro. The model of vaccinia infection of immunosuppressed mice, described in Chapter 3, provided information about the differentiation pathway of CTL, requirements for T cell activation and the need for T-T collaboration.

Recognition of antigen is the first stage of T lymphocyte activation. Interaction of specific antigen receptors with antigen and MHC molecules leads to the expression of receptors for IL-2 (Robb et al. 1981 and 1984, Smith and Cantrell, 1985). These activated cells can be induced to differentiate into effector CTL following exposure to IL-2 (Lalande et al. 1980; Gromo et al. 1987).

Studies in this thesis found that a relative insufficiency of IL-2 can abrogate the differentiation of CTLp in vivo (Chapter 3). The lack of IL-2 mediated maturation, and perhaps proliferation, of the precursors is responsible for the unresponsiveness of CTL. The conclusions are in accord with the results of Farrar and colleagues (1982) and Erard and colleagues (1985) who studied the minimal requirements for the growth and differentiation of lectin stimulated CTLp. The results also explain why administration of IL-2 to mice augments anti-viral immunity (Rouse et al. 1985; Hefeneider et al. 1983), but they are inconsistent with the idea that IL-2 does not have a role as T cell growth factor in vivo (Bandeira et al. 1987). The latter group found that naturally activated T lymphocytes (separated by cell size) failed to proliferate in the presence of recombinant IL-2. However their conclusion does not consider at least two published results: firstly, that cell size might not be the best criterion to distinguish primed from unprimed cells, as memory T cells

could be small lymphocytes (MacDonald et al. 1975); secondly: the sensitivity with which a T cell responds to IL-2 varies with the state of activation (Ashwell et al. 1986). Furthermore, recently primed T cells become transiently refractory to IL-2 after exposure to antigen or mitogen (Cantrell and Smith, 1983; Gullberg and Smith, 1986; Churilla and Braciale, 1987).

Thus, the experiments in Chapter 3 support the idea that development of the primary anti-viral CTL response in vivo is IL-2 dependent. These findings do not exclude the possibility that the early phase of development of CTLp is IL-2 independent, or at least T helper cell independent according to Sprent and colleagues (1986a), or that other factors are also necessary, since the CTL response was restored only partially by IL-2. It has been reported recently, that IFN γ and IL-4 might be also important in T cell activation. IFN-γ augments CTL response indirectly, by increasing the expression of MHC molecules (Wong et al. 1983, 1984 a & b; Steeg et al. 1982; King and Jones, 1983) and also by synergising with the effect of IL-2 (Klein and Bevan, 1983; Simon et al. 1986). It might also have a direct effect on T cell differentiation (Chen et al. 1986). In addition, a role for IL-4 in the development of the primary alloreactive CTL response has been proposed recently (Widmer and Grabstein, 1987; Rocha and Bandeira, 1988). The precise role of these factors in the development of an anti-viral T cell response has to be established.

The observation that the frequency of pCTL is not greatly affected by cyclophosphamide treatment, but that it is the development of these precursor cells which depends on the presence of IL-2 (Chapter 3) also raises the question of the necessity for T-T collaboration during the primary CTL response. From the experimental results it seems likely that the $CD8^+CD4^-$ population is not able to produce sufficient IL-2 (and maybe other factors) necessary for proliferation and differentiation, thus these processes are dependent on the function of $CD8^-$ $CD4^+$ cells. This is comparable with the conclusions of others who found that T-T collaboration is necessary in primary alloreactive and anti-viral CTL- responses. However some experiments indicate that collaboration between T cell subsets is not always necessary (see Chapter 1, section 4.3). Although $CD8^+$ cells are thought to be capable of producing some IL-2 (Andrus et al. 1981; Kelso and Glasebrook, 1984; Heeg et al. 1987) although this is disputed (Rocha and Bandeira, 1988) and there are some $CD8^+$ T cell clones which do not need and do not produce detectable amount of IL-2 but are able to proliferate upon antigenic stimulation (Moldwin et al.

1986; Widmer and Bach, 1981), the majority of CTL seem to require IL-2 production by $CD4^+$ cells. Clonal amplification of the CTL response during acute viral infection, an IL-2 dependent process, is probably necessary to combat infection. Although the experiments in this thesis did not address the question of whether CTL differentiation in vivo involves cell proliferation, the results of LDA studies (Chapters 3 and 6) suggest that the effector cells develop in this way: the frequency of virus-specific CTLp in normal mouse spleen was found to be about 1/30000 - 1/60000 which increases to about 1/1000 by 7 days after infection. Similar frequencies have been found by others (Owen et al. 1982; Moskophidis et al. 1987) and must involve clonal expansion. The published results in this field are rather controversial: previous in vitro studies have concluded that cell proliferation usually occurs during CTLp differentiation . However inhibition of cell-division with cytosine-arabinoside does not abolish the cytotoxic activity induced in a 24 hr MLC (MacDonald and Lees, 1980; Lefrancois et al. 1985). These studies seem to support the hypothesis of Mitchison and Petterson (1983) that derepression of genetic information is taking place instead of proliferative differentiation. In vivo experiments (Kimura and Wigzell, 1983) provided evidence that during allograft reaction in vivo CTL arise via a mechanism circumventing blast formation and cellular proliferation. An explanation for these observations is that there are a high number of precursors present. However Denizot et al. (1986) came to the opposite conclusion and were also using an in vivo allogeneic system: they found specific, proliferating $Ly\ 2^+$ conjugate-forming CTL in mouse peritoneal cavity 10 days after injection of allogeneic tumor cells. Furthermore, in LCMV-infected mice relatively low doses of Cy administered 2-5 days after the virus, completely abrogated the effector function of CTL (Allan and Doherty, 1985). As Cy preferentially kills dividing cells, this experiment strongly suggests that cell proliferation is an important part of CTL differentiation during primary anti-viral immune response.

The major part of this thesis deals with in vivo primed, virus-immune memory CTL. From previously reported studies it has been known that these cells are quantitatively and qalitatively different from those in unprimed mice (MacDonald, 1982). The quantitative difference is manifes as an increased precursor frequency (Ryser and MacDonald, 1979), while the qualitative changes can be described as increased avidity of the antigen receptors which can be measured by the

level of resistance to inhibition of CTL-activity with anti-CD8 antibody (MacDonald et al. 1982). The results in Chapters 4 and 5 add two more points to the characterization of memory T cells as will be discussed below.

Previous studies on memory T cells have investigated mainly in vitro primed, alloreactive CTL. These cells, derived from long-term MLC, become small and cytolytically inactive after 9 days of the culture (MacDonald et al. 1975). They re-express specific cytolytic activity upon restimulation with the original antigen (MacDonald et al. 1975). Proliferation of CTL occurs reaching a peak after 3-4 days. Supernatants derived from restimulation of primary alloreactive cultures can also cause re-expression of cytolytic activity (Ryser et al. 1978; Wagner and Rollinghoff, 1978). Purified IL-2 in the absence of added antigen can also induce in vitro - (Ochoa et al. 1986) and in vivo - primed (Lefrancois et al. 1984) memory CTL to re-express cytolytic activity.

It has been demonstrated by Isakov and Altman (1985) that PMA and Cal stimulates IL-2R expression, IL-2 production and cytolytic activity of 14 day primary MLR cells, but not the cytolytic activity of $CD8^+$ clones. Truneh et al. (1985) found that T lymphocytes from mice primed with allogeneic cells can proliferate and lyse allogeneic target cells after in vitro stimulation with ionomycin plus phorbol ester. Based on the results in Chapters 4,5 and 6 it can be concluded that CTL primed in vivo with foreign MHC or non-MHC antigens can be stimulated to proliferate with low concentrations of PMA and Cal, increase expression of IL-2 receptor and to express highly specific cytotoxic activity in the presence of IL-2. This method of the stimulation is a convenient procedure for restimulation in the absence of antigen and accessory cells. However,it also has the potential to distinguish between primed and unprimed cells, as cytotoxic activity cannot be induced by this protocol from unprimed CTL even if they are present at a relatively high frequency, as is the case with alloreactive cells. The explanation for this difference between alloreactive and primed anti-viral CTL would need a more sophisticated analysis to allow comparison of the pathways of signal transduction, or targets for phosphorylation,in cells activated by different procedures.

The results in Chapter 5 concerning the IL-2 dependent development of the secondary CTL response are in agreement with the findings of others (see above). The lymphokine is produced by $CD4^+$ cells, depletion of this population completely abolished the development of cytotoxic

activity, but not the proliferation, of the $CD8^+$ population. In the presence of IL-2, antibody to the IL-2 receptor completely blocked the proliferation of the undepleted cells. It would be worth examining whether the proliferation of the $CD8^+$ subset in the absence of detectable cytotoxic activity is IL-2 independent or whether the $CD8^+$ cells are able to produce some IL-2 and (perhaps) other factors, which would support only the proliferation but not the differentiation of these cells.

The similarities between in vitro and in vivo primed lymphocytes in the requirements for, and characteristics during, activation suggested that T cells isolated from virus-primed mice might be identified by expression of the $Ly24^+$ antigen which has been shown to be expressed on T cells following in vitro stimulation (Lynch et al. 1987). The experiments (Chapter 5) have shown that virus-immune memory T cells are enriched in the $Ly24^+$ subset. The findings are in accord with the results of Budd et al. (1987 a and b) who have shown that alloimmunization, priming with Moloney sarcoma virus (MSV) or immunization with H-Y antigen greatly enriched the antigen-specific CTLp in the $Ly24^+$ population. A comparable situation may apply in man,in that T cells enriched for the Ta_1 antigen have the potential to respond to tetanus toxoid and mumps antigens in bulk culture (Hafler et al. 1986). Intrestingly Ta_1, like Ly24, is stably expressed by activated T cells and its molecular weight is similar to murine Ly 24 (Fox et al. 1984).

However the experiments in Chapter 5, and also those published by Budd et al. (1987 a and b) were performed only with spleen cells from one strain of mice (B6). Some results suggest, that the use of Ly 24 antigen to identify in vivo generated memory cells phenotypically may be restricted by mouse strain (Lynch, unpublished).

The results obtained from restimulation of T cells from mice primed with a recombinant vaccinia virus containing the $H\text{-}2K^d$ gene (Chapter 6) suggest that distinct T cell clones are responsible for self-MHC-restricted anti-viral and anti-allo killing,. The experiment raises the question of how the antigens are presented: is the K^d molecule presented as the intact glycoprotein (von Boehmer et al. 1979; Weiss et al. 1980; Braciale et al. 1981), or is it fragmented and expressed in the context of self-MHC molecules, as for viral (Townsend et al. 1985) and HLA (Maryanski et al. 1986; Pala et al. 1988) antigens? One way of answering this question would be to analyse the T cell repertoire of mice primed with the recombinant vaccinia virus by isolating

large number of T cell clones, or to immunoprecipitate and identify the molecules expressed on the surface of cells infected with the virus-complex. Another possibility is to see if H-2 negative F9 target cells are lysed by CTL specific for vaccinia virus + H-2K^d when infected with the recombinant vaccinia virus.

In conclusion, the data presented in this study have shown that development of primary and secondary anti-viral CTL response is an IL-2 dependent process, involves T-T interaction and results in the appearance of primed or memory T cells which are at a much higher frequency than unprimed virus-immune CTLp, and differ from these: a) in requirements for the stimulation of cytotoxic activity, b) in the appearance of a distinct surface marker, Ly 24.

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